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13. SUPPLEMENTARY NOTES

14. ABSTRACT In vitro colony formation studies demonstrated that UCN-01 and the MEK1/2 inhibitor PD184352 interacted to synergistically kill human mammary carcinoma cells (MDA-MB-231, MCF7). Athymic mice were implanted in the rear flank with either MDA-MB-231 or MCF7 cells and tumors permitted to form to a volume of ~100 mm^3 prior to a two day exposure of either Vehicle, PD184352 (25 mg/kg), UCN-01 (0.1-0.2 mg/kg) or the drug combination. Tumor volume was measured every other day and tumor growth determined over the following ~30 days. Transient exposure of MDA-MB-231 tumors or MCF7 tumors to either PD184352 or UCN-01 did not alter tumor growth rate or the mean tumor volume in vivo 30 days after drug administration. In contrast, combined treatment with PD184352 and UCN-01 significantly reduced MDA-MB-231, and largely abolished MCF7 tumor growth. Tumor control values for both cell lines were 0.36. Collectively, these findings argue that UCN-01 and MEK1/2 inhibitors have the potential to suppress mammary tumor growth in vivo which is independent of estrogen dependency.

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Introduction.

The discovery of multiple intracellular signaling pathways in the late 1980's and early 1990's has increased our knowledge of how growth factors control cell growth, as well as how DNA damage can cause a survival-response of cell cycle arrest. Thus interruption of the pathway which causes DNA damage-induced cell cycle arrest, by drugs such as UCN-01 and caffeine, leads to increased radio- and chemo-sensitivity of tumor cells. In addition, inhibition of growth factor signaling pathways, such as the phosphatidyl 3-inositol kinase (PI 3-kinase) and mitogen activated protein kinase (MAPK) pathways, can also lead to enhanced radio- and chemo-sensitivity. These concepts have been reported by many groups and are generally accepted within the field of experimental therapeutics. The majority of studies have been performed in vitro. Combinatorial in vivo animal studies using drugs which modulate signaling and radiation, and Phase I clinical trials, are at a more preliminary stage. In contrast to the now standard concept that "a signaling modulator plus DNA damaging agent" will enhance cell killing by the DNA damaging agent, we have discovered that two signaling modulators can interact in a novel manner to cause profound cell killing, which is further enhanced by a DNA damaging agent. Specifically, we discovered that when a diverse range of tumor cells were treated in vitro with an inhibitor of the G2/M cell cycle checkpoint (UCN-01), the cyclin dependent kinase Cdc2 and the MAPK pathway became activated. Inhibition of the MAPK pathway by MEK1/2 inhibitors promoted further activation of Cdc2 and subsequently a large synergistic increase in cell killing. The killing effect was magnified in a supra-additive fashion in colony formation assays when drug combination treated cells were simultaneously exposed to ionizing radiation. The concept that disruption of a critical cell cycle control pathway (Chk1/Cdc25C/Cdc2) causes compensatory activation of a critical survival pathway (Raf-1/MEK/ERK) is novel and was unexpected. Inhibition of the compensatory MAPK survival signal causes profound killing in tumor cells, but notably, not in non-transformed cell types. The central goal of this application is to translate our novel in vitro findings (funded by DAMD 99-1-9426) into an in vivo xenograft athymic mouse system.

Body.

The central hypothesis of this proposal is that the novel drug combination of a cell cycle checkpoint abrogator (UCN-01) and a MEK1/2 inhibitor (PD98059, U0126, PD184352) will increase the morbidity of human mammary carcinoma cells, grown as tumors, in vivo. The proposal has the following Specific Aims:

Aim 1. : Does the combination of UCN-01 and MEK1/2 inhibitors alter mammary tumor cell growth and survival in vivo?

Aim 2. : Does exposure of [UCN-01+MEK1/2 inhibitor] treated tumors to ionizing radiation further reduce tumor size and re-growth potential, and increase cell killing?

We have demonstrated that the MEK1/2 inhibitor PD184352, at a clinically achievable plasma concentration (25 mg/kg, injected I.P., once every 8h for 48h) combined with the Chk1-PKC inhibitor UCN-01 at a clinically achievable concentration (0.1-0.2 mg/kg, injected I.P., once every 24h for 48h) resulted in a significant reduction in tumor growth for up to 35 days post-drug administration (Figures 1 and 2). In contrast to the combined effects of the drugs, each drug individually had no effect on the growth parameters of treated tumors. This study has been repeated, with similar results, three times.

Figure 1. Combined exposure of MDA-MB-231 invasive mammary carcinoma tumors to UCN-01 and PD184352 results in a greater than additive suppression of tumor growth in vivo. Athymic mice were injected in their flanks with 10^7 MDA-MB-231 tumor cells and tumors permitted to form over the next two weeks. In two weeks, tumors of volume 100-150 mm^3 have

developed ("day 0"). At day 0, animals were injected with vehicle (10% (v/v) DMSO in PBS), MEK1/2 inhibitor (PD184352, 25 mg/kg, injected I.P., once every 8h for 48h), UCN-01 (0.2 mg/kg, injected I.P., once every 24h for 48h) or MEK1/2 inhibitor and UCN-01 combined. Tumor volume was assessed initially on a daily basis and then every other day. Each data point represents the mean percentage increase in tumor volume from 6 animals (tumors).

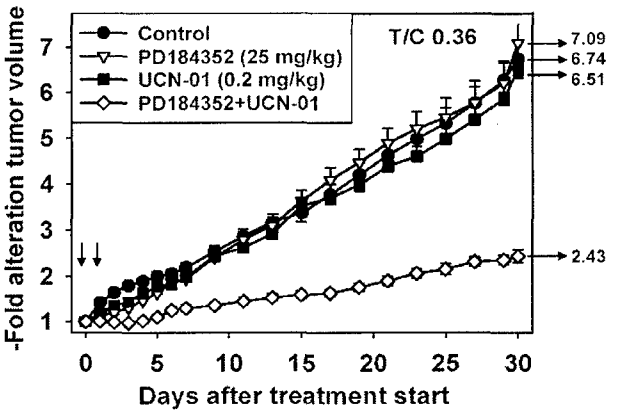
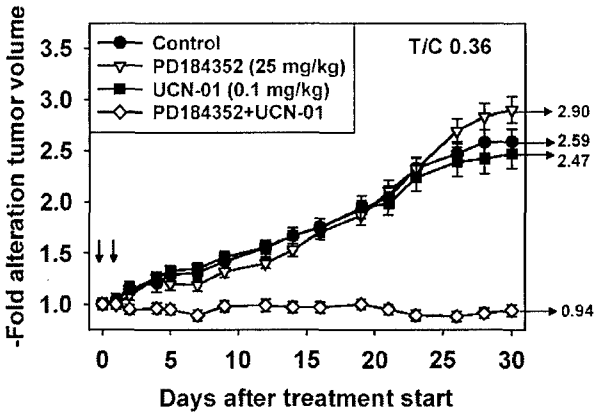


Figure 2. Combined exposure of MCF7 mammary carcinoma tumors to UCN-01 and PD184352 results in a greater than additive suppression of tumor growth in vivo. Athymic mice were injected in their flanks with 10^7 MCF7 tumor cells and tumors permitted to form over the next two weeks. In two weeks, tumors of volume 100-150 mm^3 have developed ("day 0"). At day 0, animals were injected with vehicle (10% (v/v) DMSO in PBS), MEK1/2 inhibitor (PD184352, 25 mg/kg, injected I.P., once every 8h for 48h), UCN-01 (0.1 mg/kg, injected I.P., once every 24h for 48h) or MEK1/2 inhibitor and UCN-01 combined. Tumor volume was assessed initially on a daily basis and then every other day. Each data point represents the mean percentage increase in tumor volume from 8 animals (tumors).



As can be seen from the tumor growth data, a two-day exposure to either PD184352 or UCN-01 had no measurable differential impact on tumor growth within 4 days after exposure, suggesting that a 2 day exposure of established tumors to growth-modulatory protein kinase inhibitors results in initial tumor growth suppression due to the presence of the drug (as one would expect), followed by a modest enhancement of the tumor cell growth rate after cessation of drug treatment, which is transient. In contrast to the effects of the individual drugs, combined treatment with MEK1/2 and UCN-01 resulted in growth suppression for 5 days, and a slower growth rate of the tumor thereafter. Based on the calculations of our biostatistician, the rate of tumor growth in combined drug treated cells was significantly less than that observed in either vehicle, MEK1/2 inhibitor alone or UCN-01 alone. The overall slope (quadratic) portion of the

growth curve for the tumors treated with the drug combination was also significantly different compared to the other treatment conditions.

Based on these tumor growth rate findings, at day ~32, tumors were isolated, macerated and digested with trypsin. Isolated tumor cells were counted and plated at varying densities to determine in vitro colony formation.

Figure 3. Combined exposure of MDA-MB-231 tumors in vivo to UCN-01 and PD184352 results in a greater than additive suppression of tumor cell colony forming ability in vitro. Tumors were treated with kinase inhibitor drugs for 2 days. Thirty five days after drug exposure, tumors were isolated, macerated and digested with trypsin. Isolated tumor cells were counted and plated at varying densities to determine the colony formation potential of these isolated tumor cells in vitro. Colony formation was determined 10 days after plating. Data are plotted with respect to the impact of PD184352 on cell survival, thus vehicle control and UCN-01 colony formation are both defined as 1.00.

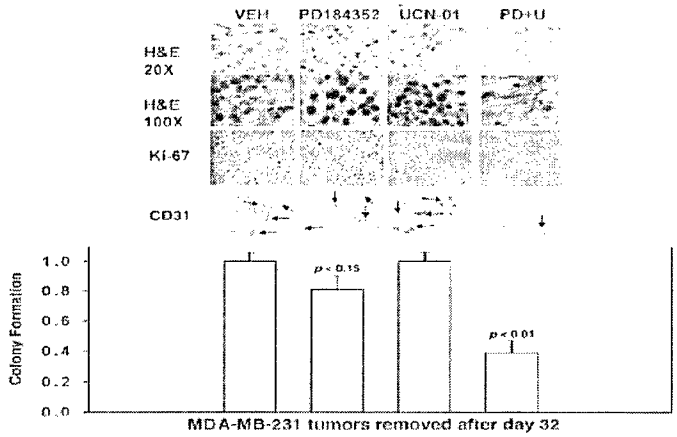
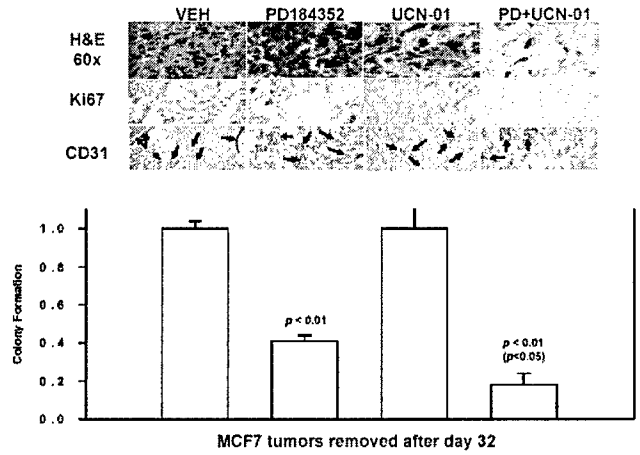


Figure 4. Combined exposure of MCF7 tumors in vivo to UCN-01 and PD184352 results in a greater than additive suppression of tumor cell colony forming ability in vitro. Tumors were treated with kinase inhibitor drugs for 2 days. Thirty five days after drug exposure, tumors were isolated, macerated and digested with trypsin. Isolated tumor cells were counted and plated at varying densities to determine the colony formation potential of these isolated tumor cells in vitro. Colony formation was determined 10 days after plating. Data are plotted with respect to the impact of PD184352 on cell survival, thus vehicle control and UCN-01 colony formation are both defined as 1.00.



As can be seen above, the ability of tumor cells treated with PD184352 and UCN-01 in vivo to then grow in vitro is significantly less (asterisk) than those treated with either drug individually.

More of note is that the growth potential of tumor cells treated with PD184352 and UCN-01 one month prior to isolation is still significantly lower than the growth potential of tumor cells treated with vehicle or with either drug individually. This suggests that in addition to killing tumor cells in vitro, as shown by McKinstry et al. Cancer Biology and Therapy (2002) 1: 243-253, the combination of a Chk1 inhibitor and a MEK1/2 inhibitor in vivo also alters the log-term growth properties of tumor cells. Additional studies on sections of treated tumors have determined the incidence of apoptosis, Ki67 levels and vascularization. These studies are included in the attached appendix manuscript, recently submitted to Clinical Cancer Research.

Key Research Accomplishments.

(1). MEK1/2 inhibitors and Chk1 inhibitors interact to suppress the growth of established mammary tumors in vivo. This is effect is independent of p53 status, estrogen dependency, caspase 3 levels or oncogenic K-RAS expression.

(2). MEK1/2 inhibitors and Chk1 inhibitors interact to suppress the in vitro colony formation of isolated cells from mammary tumors that were treated one month previously with the kinase inhibitor drugs.

Reportable Outcomes.

MEK1/2 inhibitors and Chk1 inhibitors interact to suppress the growth of established mammary tumors in vivo. MEK1/2 inhibitors and Chk1 inhibitors interact to suppress the in vitro colony formation of isolated cells from mammary tumors that were treated one month previously with the kinase inhibitor drugs.

Conclusions.

Previously, we had demonstrated that MEK1/2 inhibitors and Chk1 inhibitors synergized to kill mammary carcinoma cells in vitro. Our studies over the last two years have confirmed that we can obtain similar data in vivo using MEK1/2 inhibitors and Chk1 inhibitors, and has resulted in a submitted manuscript.

So what: MEK1/2 inhibitors and UCN-01 are in the clinic in Phase II and Phase III trials, respectively; successful completion of these studies may permit the initiation of a Phase I trial for the drug combination. Our data (Figures 1-4) was provided to CTEP (National Cancer Institute) for their evaluation of our findings prior to manuscript submission (UCN-01 was supplied by CTEP, and we synthesized our own PD184352). Pfizer (the owner of PD184352) has not in the past collaborated extensively with CTEP and to the best of our knowledge, they have not responded in an affirmative manner to CTEP. However, we have obtained from CTEP a clinically active farnesyltransferase inhibitor (proprietary of Johnson & Johnson), an inhibitor of RAS signaling which is an upstream regulator of MEK1/2, and which also synergizes with UCN-01 to kill mammary carcinoma cells. We are presently determining whether farnesyltransferase inhibitor and UCN-01 interact in vivo to kill mammary tumor cells, which could then potentially be translated into the clinic. Studies combining PD184352 and UCN-01 treatment with ionizing radiation exposure would be initiated after the farnesyltransferase inhibitor and UCN-01 studies.

Transient exposure of mammary tumors to PD184352 and UCN-01 causes tumor cell death in vivo and prolonged suppression of tumor re-growth.

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Key words: MEK1/2, cell death, tumor control.

Running Title: Tumor growth inhibition

Abbreviations: UCN-01: 7-hydroxystaurosporine; ERK: extracellular regulated kinase; MEK: mitogen activated extracellular regulated kinase.

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Abstract.

Previous studies from our group have demonstrated in vitro that UCN-01 (7-hydroxystaurosporine) and inhibitors of MEK1/2 interact to cause tumor cell death in a wide variety of malignant, but not in non-transformed, cell types. The present studies determined whether UCN-01 and MEK1/2 inhibitors interacted to cause tumor cell death in vivo. In vitro colony formation studies demonstrated that UCN-01 and the MEK1/2 inhibitor PD184352 interacted to synergistically kill human mammary carcinoma cells (MDA-MB-231, MCF7) with similar combination index values. Athymic mice were implanted in the rear flank with either MDA-MB-231 or MCF7 cells and tumors permitted to form to a volume of ~100 mm^3 prior to a two day exposure of either Vehicle, PD184352 (25 mg/kg), UCN-01 (0.1-0.2 mg/kg) or the drug combination. Tumor volume was measured every other day and tumor growth determined over the following ~30 days. Transient exposure of MDA-MB-231 tumors or MCF7 tumors to either PD184352 or UCN-01 did not significantly alter tumor growth rate or the mean tumor volume in vivo ~15-30 days after drug administration. In contrast, combined treatment with PD184352 and UCN-01 significantly reduced MDA-MB-231, and largely abolished MCF7 tumor growth. Tumor control values for both cell lines were 0.36. Tumor cells isolated ~30 days after combined drug exposure exhibited a significantly greater reduction in plating efficiency using ex vivo colony formation assays than tumor cells that were exposed to either drug individually. Reduced tumor growth correlated with profound tumor cell death within 5 days of combined drug exposure, which was also evident ~30 days after exposure. In addition, tumor cell death correlated with a reduction in the phosphorylation of ERK1/2 and the immuno-reactivity of Ki67 and of CD31. Collectively, these findings argue that UCN-01 and MEK1/2 inhibitors have the potential to suppress mammary tumor growth in vivo which is independent of p53 status, estrogen dependency, caspase 3 levels or oncogenic K-RAS expression.

Introduction.

UCN-01 (7-hydroxystaurosporine) is currently being evaluated as an antineoplastic agent in clinical trials, both alone and in combination with chemotherapeutic agents and ionizing radiation [1]. UCN-01 exerts anti-proliferative activity both *in vitro* and *in vivo*, an action which may be related to inhibition of protein kinase C (PKC) isoforms [2]. UCN-01 also enhances the cytotoxicity of chemotherapeutic agents by several postulated mechanisms, including inhibition of Chk1 [3]. Inhibition of Chk1 may directly promote activation of the protein phosphatase Cdc25C and can also interfere with Cdc25C elimination by blocking its binding to 14-3-3 proteins and subsequent degradation [3]. Down-regulation of Cdc25C results in enhanced phosphorylation and inactivation of cyclin-dependent kinases (Cdks) such as $p34^{cdc2}$, which are critically involved in G2/M cell cycle arrest following DNA damage [4]. More recently, UCN-01 has also been shown to inhibit the downstream effector of PI3 kinase, PDK-1, in the same concentration range as PKC isoforms [5]. Thus, UCN-01 can function as a checkpoint abrogator capable of enhancing the lethal actions of DNA-damaging agents, including ionizing radiation [6], cisplatin [7], Ara-C [8] and campothecins [9]. It has generally been thought that UCN-01, administered at pharmacologically achievable concentrations, promotes cell death *via* Cdk dephosphorylation rather than by inhibition of PKC enzymes and PDK-1 [10].

The ability of the ERK1/2 pathway to regulate proliferation *versus* differentiation and survival appears to depend upon the amplitude and duration of ERK1/2 activation. A short activation of the ERK1/2 cascade has been correlated with enhanced progression through the G1-S transition [11]. In contrast, prolonged elevation of ERK1/2 activity has been demonstrated to inhibit DNA synthesis through super-induction of the Cdk inhibitor protein $p21^{CIP1}$, which may in turn lead to differentiation or to cell death [12]. In addition to a role for ERK1/2 signaling in the G1/S phase transition, it has also been argued that ERK1/2 signaling may be involved in the ability of cells to progress through G2/M, particularly following DNA damage-induced growth arrest [13,14].

Multiple intracellular signal transduction pathways e.g. ERK1/2 and PI3K/AKT are often highly activated in tumor cells, and have been proposed as therapeutic targets in preventing cancer cell growth in many malignancies [e.g. 15-17]. Furthermore, inhibition of chemotherapeutic drug and radiation -induced growth factor receptor or signaling pathway activation by novel inhibitors of kinase domains has been shown by many groups to enhance the toxicity of established chemotherapy / radiation modalities [18-21]. An alternative approach, however, to killing tumor cells without using established cytotoxic therapies is to exploit their reliance (i.e. addiction) to high levels of signaling pathway activity to maintain growth and viability. Thus previous studies by this group have demonstrated that, UCN-01, at clinically relevant concentrations in vitro causes activation of the ERK1/2 pathway in transformed cell types. Prevention of ERK1/2 pathway activation, by inhibition of either MEK1/2 or RAS function, rapidly promoted UCN-01 -induced tumor cell death in a synergistic fashion [22-26]. Non-transformed cells from multiple tissues were noted in several studies to be insensitive to apoptosis-induction by this strategy. The overall concept that blocking compensatory survival pathway activation responses leads to tumor cell killing has been extended by our group and others using a variety of small molecule inhibitors of kinases and other enzymes, including: e.g. flavopiridol and PI3 kinase inhibitors; flavopiridol and histone deacetylase inhibitors; histone deacetylase inhibitors and perifosine; MEK1/2 inhibitors and imatinib mesylate [27-30].

The present studies were designed to determine whether MEK1/2 inhibitors and UCN-01 interacted in a synergistic fashion in vivo to suppress mammary tumor cell growth in vivo. Our findings demonstrate that UCN-01 and MEK1/2 inhibitors potently inhibit mammary tumor growth in vivo an effect that is independent of p53 status, estrogen dependency, caspase 3 expression or oncogenic K-RAS expression.

Materials and Methods.

Materials. Phospho-/total-ERK1/2 and anti-BAX antibodies were purchased from Cell Signaling Technologies (Worcester, MA). The anti-BAX monoclonal antibody 6A7 for immunoprecipitation of conformationally changed BAX was from Sigma-Aldrich (St. Louis, MO). The anti-Ki67 antibody was purchased from Oncogene Research Products (San Diego CA), anti-CD31 / PECAM antibody was purchased from (Santa Cruz Biotechnology, Santa Cruz CA) and the cleaved caspase 3 antibody was purchased from Cell Signaling Technologies (Worcester, MA). All the secondary antibodies (anti-rabbit-HRP, anti-mouse-HRP, and anti-goat-HRP) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Enhanced chemiluminescence (ECL) and TUNEL kits were purchased from NEN Life Science Products (NEN Life Science Products, Boston, MA) and Boehringer Mannheim (Manheim, Germany), respectively. Trypsin-EDTA, RPMI, penicillin-streptomycin were purchased from GIBCOBRL (GIBCOBRL Life Technologies, Grand Island, NY). PD98059 and U0126 were purchased from Calbiochem / EMD sciences (San Diego, CA). UCN-01 was kindly provided by Dr. Janet Dancey, National Cancer Institute, Developmental Therapeutics Program / CTEP. PD184352 was chemically synthesized in house based on the published structure of the drug and stored as a powder in a dry nitrogen atmosphere under light-protected conditions at -80°C. Other reagents were as described in [22-26].

Methods.

Culture and in vitro exposure of cells to drugs. MDA-MB-231 and MCF7 cells were cultured at 37 °C (5% (v/v CO_2) *in vitro* using RPMI supplemented with 10% (v/v) fetal calf serum. *In vitro* UCN-01 / PD184352 / U0126 / PD98059 treatment was from a 100 mM stock solution of each drug and the maximal concentration of Vehicle (DMSO) in media was 0.02% (v/v).

In vitro and ex vivo cell treatments, SDS-PAGE and Western blot analysis. For in vitro analyses of short-term apoptosis effects, cells were treated with Vehicle, UCN-01, PD184352 / U0126 / PD98059 or their combination for the indicated times. For apoptosis assays, cells were pre-treated with IETD and LEHD (each, 20 μM) as

described [22]; cells were isolated, fixed to slides, and stained using a commercially available TUNEL assay kit according to manufacture's instructions [22]. For cell cycle analyses, cells were treated with drugs and 48h after exposure were isolated, fixed in 70% (v/v) ethanol, treated with RNAse and propidium iodide overnight at 4 °C, subjected to flow cytometery and data processed using Verity Winlist software.

Cells for *in vitro* or *ex vivo* colony formation assays were plated at 250-4000 cells per well in sextupilcate and for *in vitro* assays 14 hours after plating were treated with either Vehicle (DMSO), UCN-01 (10-150 nM), PD184352 (0.1-2.0 μM) or the drug combination for 48h followed by drug removal. Ten-14 days after exposure or tumor isolation, plates were washed in PBS, fixed with methanol and stained with a filtered solution of crystal violet (5% w/v). After washing with tap water, the colonies were counted both manually (by eye) and digitally using a ColCount™ plate reader (Oxford Optronics, Oxford, England). Data presented is the arithmetic mean (± SEM) from both counting methods from multiple studies. Colony formation was defined as a colony of 50 cells or greater.

For SDS PAGE and immunoblotting, cells were plated at 5 x 10^5 cells / cm^2 and treated with PD184352 / PD98059 and UCN-01 at the indicated concentrations and after the indicated time of treatment, lysed for immunoprecipitations of BAX [23, 24], or with whole-cell lysis buffer (0.5 M Tris-HCl, pH 6.8, 2%SDS, 10% glycerol, 1% β-mercaptoethanol, 0.02% bromophenol blue), and the samples were boiled for 30 min. The boiled samples were loaded onto 10-14% SDS-PAGE and electrophoresis was run overnight. Proteins were electrophoretically transferred onto 0.22 μm nitrocellulose, and immunoblotted with various primary antibodies against different proteins. All immunoblots were visualized by ECL.

In vivo exposure of mammary carcinoma tumors to drugs. Athymic female NCr-nu/nu mice were obtained from Jackson Laboratories (Bar Harbor, ME). Studies were performed independently by three operators. Mice were maintained under pathogen-free conditions in facilities approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulations and standards of the U.S. Department of Agriculture, Washington, DC, the U.S. Department of Health and Human Services, Washington, DC, and the

National Institutes of Health, Bethesda, MD. For generation of MCF7 tumors, 8 week old female athymic mice were implanted in the midline below the scapulae with 90 day slow release estrogen pellets (Innovative Research #NE-121; 0.72mg 17-β-estradiol). MDA-MB-231 and MCF7 cells were cultured and isolated by trypsinization followed by cell number determination using a hemacytometer. Cells were resuspended in phosphate buffered saline and five million tumor cells per 100 µl PBS were injected into the right rear flank of each mouse, and tumors permitted for form to a volume of ~100 mm^3 over the following 3-4 weeks. PD184352 was aliquoted into ~50mg / vial and stored in a -20°C cold room under vacuum and protected from light. For animal administration, PD184352 was first dissolved in DMSO; the amount of µl of DMSO used was 4 times the number mgs of PD184352 to be dissolved (i.e. 50mg in 200 µl DMSO). Next, an equal volume of Cremophor (Sigma, St. Louis, MO) was added. After mixing, a 1:10 dilution was made with 0.9% sterile saline. Animals were I.P. injected with PD184352 to a final concentration of 25 mg/kg body mass. Animals received two more injections of PD184352, eight hours apart for 2 days. UCN-01 was administered 45min after the first PD184352 injection each day for two days. UCN-01 was diluted in a solution of 2% (w/v) Na citrate pH 3.5. An aliquot of diluent was filter sterilized using a 10 ml syringe and 0.2 µM filter. The UCN-01 was further diluted 1:20 with Na citrate, and animals were injected I.P. for a final concentration of 0.2 mg/kg (MDA-MB-231) or 0.1 mg/kg (MCF7). Each animal not receiving a dose of PD184352 or UCN-01 at the time of drug treatment was given an I.P. injection of diluent alone in an volume equal to the amount given with the drug. Tumor volumes were calculated from the formula ($l \times w^2$) / 2, where l and w are longest and shortest lengths, respectively, of the tumor. Tumor growth was expressed as relative –fold change in tumor volume, ($T\chi$ / T_0), where T is the mean tumor volume of all tumors at a particular time in days χ and T_0 was the mean tumor volume at day 0.

Ex vivo manipulation of mammary carcinoma tumors Animals were euthanized by CO_2 and placed in a BL2 cell culture hood on a sterile barrier mat. The bodies of the mice were soaked with 70% (v/v) EtOH and the skin around the tumor removed using small scissors, forceps and a disposable scalpel. These implements were flame sterilized between removal of the outer and inner layers of skin. A piece of the tumor (~50% by volume) was

removed and placed in a 10 cm dish containing 5 ml of RPMI cell culture media, on ice. In parallel the remainder of the tumor was placed in 5 ml of Streck Tissue Fixative (Fisher Scientific, Middletown VA) in a 50 ml conical tube for fixation; H&E staining of fixed tumor sections was performed as described [31]. The tumor sample that had been placed in RPMI was minced with a sterile disposable scalpel into the smallest possible pieces then placed in a sterile disposable flask. The dish was rinsed with 6.5 ml of RPMI medium which was then added to the flask. A 10x solution of collagenase (Sigma, St. Louis MO; 2.5 ml, 28 U/ml final concentration) and 10x of enzyme mixture containing DNAse (Sigma, St. Louis MO; 308 U/ml final concentration) and pronase (EMD Sciences, San Diego CA; 22,500 U/ml final concentration) in a volume of 1 ml was added to the flask. The flasks were placed into an orbital shaking incubator at 37°C for 1.5 hours at 150 rpm. Following digestion, the solution was passed through a 0.4 μM filter into a 50 ml conical tube. After mixing, a sample was removed for viable and total cell counting using a hemacytometer. Cells were centrifuged at 500 x g for 4 min, the supernatant removed, and fresh RPMI media containing 10% (v/v) fetal calf serum was added to give a final resuspended cell concentration of 1×10^6 cells / ml. Cells were diluted and plated in 10 cm dishes in triplicate at a concentration of 2×10^3 cells / dish for Control, PD184352 and UCN-01 treatments and 4×10^3 / dish for combined PD184352 and UCN-01 exposure [22].

Immunohistochemistry and staining of fixed tumor sections. Fixed tumors were embedded in paraffin wax and 10 μM slices obtained using a microtone. Tumor sections were de-parafinized, rehydrated and antigen retrieval in a 10 mM (w/v) Na Citrate / Citric acid buffer (pH 6.7) heated to 90 °C in a constant temperature microwave oven. Prepared sections were then blocked and subjected to imunohistochemistry as per the instructions of the manufacturer for each primary antibody (phospho-ERK1/2; Ki67; CD31; cleaved caspase 3). Prior to incubation with the primary antibody, the endogenous peroxidase activity was blocked by a 30 min. incubation with 3.0 % (v/v) H_2O_2 in methanol. The sections were then washed in phosphate buffered saline (PBS; 3 x 20 min), incubated in the primary antibody at 1:300 dilution overnight and washed again in PBS (3 x 20 min) the next day. Slides were then incubated with a biotin-conjugated secondary antibody, which was used at a dilution of

1:500 (in goat serum) for 2h, followed by several washes in PBS (3 x 20 min). To develop the slides, an avidin-biotin-peroxidase complex (ABC reagent; Vectastain ABC Elite kit, Vector Laboratories, Burlingame, CA) kit was used, at dilutions of 1:250 "A" and 1:250 "B" in 1% (v/v) goat serum diluent for 1h. After, several washes in PBS, the sections were transfered to Tris buffered saline (TBS) and the peroxidase reactivity developed using di-aminobenzidine (DAB) substrate (1h at 37 °C), followed by several washes in TBS. The tissue sections were de-hydrated, cleared and mounted with cover-slips using Permount. The permanently mounted slides were allowed to dry overnight and were photographed at the indicated magnification. The area selected for all photo-micrographs was the proliferative zone, within 2 mm of, or juxtaposed to leading edge of the tumor.

Data analysis. Comparison of the effects of various treatments was performed using a mixed effects model with an AR(1) error covariance structure. Tumor growth data shown are the means of one representative experiment using 6-9 animals per treatment condition. Experiments were performed for each cell line at least three times with similar data. Differences with a p-value of < 0.05 were considered statistically significant. Experiments shown are the means of multiple individual points (± SEM).

Results.

Initial studies extended our preliminary observations to demonstrate that the MEK1/2 inhibitor PD184352 and UCN-01 interacted in a synergistic manner to kill mammary tumor cells in vitro [22]. A 48h exposure of either MDA-MB-231 or MCF7 cells to PD184352 and UCN-01 resulted in a synergistic induction of cell killing as measured ~14 days after drug removal in colony formation assays (Table 1 and Table 2). Of note, combined exposure of paracrine TGFα / ERBB1 –regulated MDA-MB-231 cells to the ERBB1 inhibitor AG1478 and UCN-01 surprisingly resulted in an antagonistic interaction for cell killing (Table 3). Treatment of MDA-MB-231 and MCF7 cells in vitro with UCN-01 and either of the MEK1/2 inhibitors PD184352 or U0126 promoted apoptosis (Figure 1A). Treatment of MDA-MB-231 cells in vitro with the MEK1/2 inhibitor PD98059 and UCN-01 correlated with the cleavage of pro-caspase 9, pro-caspase 8, pro-caspase 3, altered BAX conformation, and apoptosis as measured by flow cytometry and TUNEL staining (Figure 1B). Apoptosis was abolished by the pan-caspase inhibitor zVAD and partially blocked by either the caspase 8 inhibitor IETD or the caspase 9 inhibitor LEHD (Figure 1B, data not shown). In vitro, primary mammary epithelial cells, CD34+ bone marrow progenitor cells and primary rat hepatocytes were all resistant to drug combination -induced lethality (Figure 1C) [22, 23].

Athymic mice were injected in the rear flank with MDA-MB-231 or MCF7 cells and tumors permitted to form. Mice bearing ~100-150 mm^3 tumors were then injected I.P. with vehicle, PD184352, UCN-01 or the drug combination for two days. Tumor volume measured over the following ~30 days. Treatment of mice with either UCN-01 or PD184352 transiently suppressed tumor growth; however, 15-30 days following drug exposure, no significant change in tumor volume was noted comparing vehicle, PD184352 or UCN-01 treated tumors (Figure 2 and Figure 3). In contrast to data using individual drug treatments, combined treatment of animals with PD184352 and UCN-01 significantly reduced tumor growth for both mammary tumor cell types ($p < 0.05$ for MDA-MB-231; $p < 0.01$ for MCF7). Thirty days after exposure the tumor control ratio for MDA-MB-231 tumors was 0.36 and for MCF7 tumors was 0.36. These findings demonstrate that a transient 2 day combined exposure to MEK1/2 inhibition and UCN-01 results in suppression of tumor growth for up to 30 days.

As tumor growth was reduced for up to 30 days after drug exposure, additional studies examined gross tumor histology including vascularization, and cell viability ex vivo at this late time point. Tumors exposed to the drugs individually compared to vehicle control exhibited a reduced number of cells per field under H&E staining and a modest reduction in the levels of Ki67 immunoreactivity (Figure 4 and Figure 5). In contrast to our findings with individual drugs, combined exposure to PD184352 and UCN-01 caused a profound reduction in tumor cellularity and almost abolished Ki-67 immunoreactivity, indicating that tumor re-growth had been suppressed. Tumors exposed to both PD184352 and UCN-01 also appeared to have less CD31 immunoreactivity and morphology consistent with blood vessels compared to vehicle treated tumors, indicating that combined drug treatment had inhibited angiogenesis and/or killed tumor endothelial cells.

In parallel, isolated tumors were macerated, and digested to obtain individual tumor cells, that in turn were plated for ex vivo colony formation assays. Although the in vivo growth rate of MDA-MB-231 and MCF7 tumors treated individually with either PD184352 or UCN-01 was similar to tumors treated with vehicle control, the ex vivo colony formation of either PD184352 and to a lesser extent UCN-01 treated tumor cells was significantly reduced compared to control (Figure 4 and Figure 5, and associated legends). The ex vivo colony formation of cells treated in vivo with both PD184352 and UCN-01 was however significantly lower than would be expected based on the additive combination of PD184352 and UCN-01 alone. Collectively our findings argue that combined exposure to PD184352 and UCN-01 results in tumor cell killing in vivo and profoundly alters the ability of the tumor cells to proliferate in the absence of the drugs either in vivo or ex vivo.

Based on our findings in Figures 2-5 for tumor cell growth and end-point analyses, we performed additional studies to examine the response of tumors during and following cessation of drug treatment. Combined PD184352 and UCN-01 exposure resulted in a rapid induction of MDA-MB-231 tumor cell death within 2 days of treatment compared to the individual drug treatments, based on H&E staining (Figures 6A and 6B). This induction of tumor cell death in vivo correlated with increased cleavage of pro-caspase 3, a reduction in ERK1/2 phosphorylation (Figure 6C, data not shown). As was observed in vitro, treatment of MDA-MB-231

cells with UCN-01 promoted ERK1/2 activation in vivo within 24h; activation of ERK1/2 was still evident 24h following the last administration of UCN-01, however, activated ERK1/2 under these conditions appeared to be located in the nucleus (Figure 6D). Transient exposure to PD184352 or UCN-01 suppressed CD31 immunoreactivity, indicating that as individual agents the MEK1/2 inhibitor or UCN-01 caused endothelial cell death (Figure 6E). Combined exposure to PD184352 and UCN-01 suppressed CD31 immunoreactivity below that observed in tumors treated with either drug alone. Thus exposure of MDA-MB-231 tumors to PD184352 and UCN-01 results in a rapid induction of cell death that correlates with activation of the intrinsic apoptosis pathway and reduced proliferation.

Further studies then examined tumor histology ~5-20 days after drug exposure. Combined exposure to PD184352 and UCN-01, compared to either drug individually or vehicle control, resulted in a prolonged disruption of tumor cellularity as well as reduced Ki-67 immunostaining, ERK1/2 phosphorylation and CD31 immunostaining (Figure 7). Of note, in MDA-MB-231 tumors, transient exposure to UCN-01, and surprisingly PD184352, resulted in enhanced ERK1/2 phosphorylation 5-10 days following drug exposure which had dissipated to control levels by day 15 (Figure 7C). In MCF7 cells, UCN-01 treatment promoted ERK1/2 phosphorylation up to 30 days following drug exposure, which was abolished in tumors treated with UCN-01 and MEK1/2 inhibitor, as also noted for MDA-MB-231 tumor cells (Figure 8). These findings, together with those in Figure 7, demonstrate that transient exposure of mammary tumors in vivo to PD184352 or UCN-01 induces a compensatory activation of ERK1/2 which results in a transient (5-10 day) "overshoot" of ERK1/2 activity above the levels of ERK1/2 signaling in control cells, and that such a phenomenon is abolished by co-administration of these agents.

Discussion

Previous studies by this group have argued that MEK1/2 inhibitors and UCN-01 interact in vitro to promote tumor cell specific killing in a wide variety of malignancies including breast, prostate and multiple hematological cell types. The present studies were initiated to determine whether MEK1/2 inhibitors and UCN-01 interact to kill carcinoma cells in vivo.

In vivo, combined exposure of pre-formed mammary tumors to MEK1/2 inhibitor and UCN-01 resulted in a significantly greater reduction in tumor growth than either drug administered separately that correlated with cleavage of pro-caspase 3, a reduction in ERK1/2 phosphorylation and a prolonged suppression of Ki67 immunoreactivity. This observation also correlated with reduced tumor cellularity and ex vivo colony formation, 30 days after cessation of drug exposure. Tumor angiogenesis was disrupted by both individual abd by combined MEK1/2 inhibitor and UCN-01 treatment as judged by reduced CD31 staining. Collectively, these findings demonstrate that the tumoricidal properties of combined MEK1/2 inhibitor and UCN-01 treatment previously noted in vitro can be translated into several xenograft animal model systems of human breast cancer. Based on our use of MDA-MB-231 and MCF7 cells, the tumoricidal effect of MEK1/2 inhibitor and UCN-01 treatment is independent of p53 status, estrogen dependency, caspase 3 levels, or oncogenic K-RAS expression.

The net output of the cytoprotective MEK1/2 - ERK1/2 pathway has previously been shown to be a critical determinant of tumor cell survival [20, 21 and references therein]. Furthermore, activation of this cascade has been observed as a compensatory response of tumor cells to various environmental stresses, including cytotoxic drugs [20, 21]. One unanticipated finding of the present study was that in MDA-MB-231 tumors treated with UCN-01, and surprisingly also in those treated with PD184352, an increase in phospho-ERK1/2 levels was noted 5-10 days following drug administration. In MCF7 tumors, the UCN-01 mediated stimulation of ERK1/2 phosphorylation was observed up to 30 days following drug treatment. Although an increase in ERK1/2 phosphorylation in tumor cells exposed to UCN-01 has been observed by several groups following exposure to UCN-01 [22-24], a compensatory ERK1/2 response to MEK1/2 inhibition has not previously been reported.

The lack of ERK1/2 phosphorylation in MDA-MB-231 and MCF7 tumors treated with the combination of UCN-01 and PD184352 was not due to persistence of MEK1/2 inhibitor in the animals/tumors as PD184352 treated tumors showed both suppression of phospho-ERK1/2 levels during drug exposure and then a transient rebound in ERK1/2 phosphorylation above the staining intensity of control treated tumors 5-10 days following cessation of drug treatment. In addition, it would be unlikely based on published pharmacokinetic data generated in mice showing UCN-01 had a ~4-7h half-life in rodent plasma, that UCN-01 could have persisted in the MCF7 tumors for ~30 days to alter phospho-ERK1/2 levels to this time point [32, and references therein]. Thus the present findings are most consistent with the notion that tumor cells exposed to both PD184352 and UCN-01 were killed in an immediate fashion correlating with drug exposure and that following combined drug treatment, these cells were incapable of mounting a compensatory activation of the ERK1/2 pathway to either UCN-01 actions or MEK1/2 inhibition, thereby resulting in a prolonged suppression of tumor re-growth. As a two day exposure of MCF7 cells to UCN-01 elevated ERK1/2 phosphorylation for up to 30 days, our findings also suggest that transient UCN-01 exposure may modify the inherent biology of mammary tumor cells in vivo.

In addition to tumor cell killing, individual and combined treatment of tumors with PD184352 and UCN-01 also reduced CD31 immunoreactivity during and shortly following drug exposure in the tumor, arguing that either endothelial cells within the tumor vasculature were being directly killed or were being indirectly lost due to tumor cell death; of note, the reduction in CD31 reactivity was greatest in tumors treated with PD184352 and UCN-01. It is known that exposure to UCN-01 in vitro at a clinically achievable drug concentration, in vitro, can also suppress angiogenesis, although the mechanism by which this occurs has not been determined [48]. Similarly, MEK1/2 inhibitors have been noted in vitro to suppress endothelial cell proliferation [49]. One possible explanation for this observation is that in a cell type dependent manner inhibition of MEK1/2 can suppress c-Jun and AP-1 –dependent VEGF promoter activity [50]. In our system, the loss of CD31 immunoreactivity was maintained in tumors for up to 30 days after combined drug exposure, suggesting that neo-angiogenesis following drug exposure had also been inhibited. In vitro, multiple non-transformed cell types have been noted to be resistant to PD184352 and UCN-01 lethality, and additional studies will be required

to determine whether non-transformed endothelial cells in vivo, within a tumor, are susceptible to PD184352 and UCN-01 –induced cell killing.

Studies in hematological cell types have demonstrated that the lethality of UCN-01 is promoted by both MEK1/2 inhibitors and also by drugs that inhibit RAS processing; farnesyltransferase inhibitors (FTIs) [26, 33]. These studies were performed, in part, based on the assumption that RAS signaling is upstream of MEK1/2, and that upstream inhibitors of MEK1/2 function should also promote UCN-01 lethality. In general agreement with these findings, FTIs and UCN-01 also interacted in vitro to synergistically promote MDA-MB-231 and MCF7 cell death (Mitchell, Dai, Grant and Dent, Unpublished observations). In studies involving the irradiation of MDA-MB-231 tumor cells, we have noted that paracrine signaling by transforming growth factor alpha / ERBB1 upstream of ERK1/2 plays an important role in cell survival and accelerated repopulation following exposure [18, 20-22]. As both MEK1/2 inhibitors and FTIs promoted UCN-01 lethality, further studies determined whether ERBB1 inhibition could also promote UCN-01 lethality; unexpectedly, inhibition of ERBB1 suppressed UCN-01 lethality. These findings suggest that UCN-01 can promote or modify the activity of ERBB1 and that under these conditions ERBB1 is acting to promote mammary tumor cell death. These findings are not highly novel per se, as other groups in a variety of cell types have shown that ERBB1 can promote cell death via the extrinsic and intrinsic apoptotic pathways [34, 35].

In published studies treating animals with either UCN-01 or PD184352 as individual agents, noticeably higher drug concentrations than those used herein have been administered to show significant anti-tumor effects. For example, Patel *et al.* treated animals carrying head and neck tumor xenografts for five days with 7.5 mg/kg UCN-01 to achieve tumor regression [32]. Similarly, Sebolt-Leopold *et al.* and McDaid *et al.* used PD184352 concentrations in the 48-300 mg/kg range (with drug administration over each of ~14-20 days) to achieve anti-tumor effects [36, 37]. A consideration of the doses of UCN-01 and PD184352 used in the above protocols and those used in this manuscript are of importance due to the concentration ranges of each drug that are believed to be clinically achievable in patient serum. The maximal free concentration of UCN-01 in human plasma is

thought to be at or below ~100 nM with a long plasma half-life due to UCN-01 binding to human alpha 1 acidic glycoprotein, which is a considerably lower concentration than that achievable in rodent plasma [38-40]. The maximal free concentration of PD184352 in many patients based on Phase I / II clinical trail data appears to be in the range ~400 nM which is due in part to the rapid metabolism of this drug in patients [41, 42].

In our animal studies the theoretical peak UCN-01 concentration for treatment of mammary tumors with the drug, based on instantaneous absorption of the entire agent, would be in the range of ~300 nM. A 25 mg/kg dosing of PD184352 results in a theoretical peak concentration of 50 μM. Thus at least for UCN-01 the concentrations of drug used in our studies could be approached in the plasma of human patients. Notably, a newer second generation MEK1/2 inhibitor, PD0325901, has been developed which displays markedly superior pharmacokinetic characteristics in humans compared to PD184352, and it will be of great interest to determine whether this inhibitor also interacts with UCN-01 to suppress mammary tumor growth [43].

In our in vitro studies we noted that non-transformed cell types were less sensitive to the toxic effects of combined UCN-01 and PD184352 exposure than transformed cells. In vivo, we discovered that combined exposure of rodents to doses of UCN-01 and PD184352 four times greater and for twice as long as those used in Figure 3 did not cause weight loss, skin color changes or alterations in animal behavior, arguing that this drug combination is not highly toxic to animals. These findings are concordant with our in vitro data.

Breast cancer is frequently treated using estrogen receptor inhibitors (e.g. tamoxifen) [44], receptor signaling modulators (e.g. Herceptin) [45], cytotoxic chemotherapy (e.g. Docetaxel [46]) and ionizing radiation [20, 21]. In vitro studies have demonstrated that combined MEK1/2 inhibition and UCN-01 treatment radiosensitizes human mammary and prostate carcinoma cells [22]. It is also known that MEK1/2 inhibition can promote the lethality of taxanes in vitro and in vivo [37, 47]. Further studies will be required to determine whether combined PD184352 and UCN-01 treatment can further enhance the lethality of established cytotoxic therapies in vivo such as ionizing radiation or taxanes.

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Table 1. PD184352 and UCN-01 synergize in vitro to kill MDA-MB-231 cells in colony formation assays. MDA-MB-231 cells were plated in sextuplicate as single cells for colony formation assays, as described in the Methods. Cells were permitted to attach and 12h after plating and each well individually treated for 48h with the indicated concentrations of PD184352 and UCN-01. Following 48h of drug treatment, media was carefully removed, the cells washed and fresh media lacking drugs added to the cultures. Colonies were permitted to form over the following 10-14 days. Cells were fixed, stained, and counted both manually and using a Colcount^{TM} machine (Oxford Optronics, Oxford, England) [47]. The mean colony numbers from both methods of counting were used to calculate plating efficiency ± SEM (n=6) for each treatment condition. Isobologram analyses were performed using CalcuSyn software for Windows. Data are from a representative experiment (n = 3).

PD184352 (μM)	UCN-01 (nM)	Fa	CI
0.50	37.5	0.65	0.34
1.00	75.0	0.62	0.61
1.50	112.5	0.54	0.63

Table 2. PD184352 and UCN-01 synergize to kill MCF7 cells in colony formation assays. MCF7 cells were plated in sextuplicate as single cells for colony formation assays, as described in the Methods. Cells were permitted to attach and 12h after plating and each well individually treated for 48h with the indicated concentrations of PD184352 and UCN-01 (studies were performed using two different drug concentration ratios). Following 48h of drug treatment, media was carefully removed, the cells washed and fresh media lacking drugs added to the cultures. Colonies were permitted to form over the following 10-14 days. Cells were fixed, stained, and counted both manually and using a Colcount™ machine (Oxford Optronics, Oxford, England) [47]. The mean colony numbers from both methods of counting were used to calculate plating efficiency ± SEM (n=6) for each treatment condition. Isobologram analyses were performed using CalcuSyn software for Windows. Data are from a representative experiment (n = 3).

PD184352 (μM)	UCN-01 (nM)	Fa	CI
0.30	22.5	0.91	0.60
0.40	30.0	0.87	0.66
0.50	37.5	0.78	0.61
PD184352 (μM)	**UCN-01 (nM)**	**Fa**	**CI**
0.50	37.5	0.80	0.47
1.00	75.0	0.74	0.76
1.50	112.5	0.70	0.79

Table 3. AG1478 suppresses UCN-01 lethality in MDA-MB-231 cells: ERBB1 signaling has the potential to play a role in promoting UCN-01 –induced lethality. MDA-MB-231 cells were plated in sextuplicate as single cells for colony formation assays, as described in the Methods. Cells were permitted to attach and 12h after plating and each well individually treated for 48h with the indicated concentrations of AG1478 and UCN-01. Following 48h of drug treatment, media was carefully removed, the cells washed and fresh media lacking drugs added to the cultures. Colonies were permitted to form over the following 10-14 days. Cells were fixed, stained, and counted both manually and using a Colcount™ machine (Oxford Optronics, Oxford, England) [47]. The mean colony numbers from both methods of counting were used to calculate plating efficiency ± SEM (n=6) for each treatment condition. Isobologram analyses were performed using CalcuSyn software for Windows. Data are from a representative experiment (n = 3).

AG1478 (nM)	UCN-01 (nM)	Fa	CI
100	37.5	0.94	3.27
200	75.0	0.91	3.57
300	112.5	0.79	1.63

Figure Legends

Figure 1. MEK1/2 inhibitors interact with UCN-01 to synergistically promote cell death in mammary carcinoma cells that is blocked by inhibition of caspase 9 and caspase 8 function. MDA-MB-231 and MCF7 cells were cultured as described in the Methods [22]. **Panel A.** MDA-MB-231 (left panel) and MCF7 (right panel) cells were treated with Vehicle control (DMSO) or the indicated concentrations of UCN-01, PD184352 or U0126. Cells were isolated by trypsinization 48h after treatment, fixed to glass slides, and stained for double stranded DNA breaks using a TUNEL assay kit. The percentage apoptosis was determined in triplicate from two separate experiments ± SEM (*$p < 0.05$ greater than UCN-01 alone). **Panel B.** *Left section*; MDA-MB-231 cells were treated with Vehicle control (DMSO), PD98059 (25 μM), UCN-01 (150 nM) or both drugs together in the presence of either Vehicle (DMSO), the caspase 8 inhibitor IETD (20 μM), the caspase 9 inhibitor LEHD (20 μM) or both caspase inhibitors. Data shown are the mean number of apoptotic staining cells from randomly selected fields of fixed cells (n=5 per slide, 3 parallel individual experiments ± SEM; *$p < 0.05$ greater than UCN-01 alone; #$p < 0.05$ less than corresponding value in cells not treated with caspase inhibitor). The inset panel shows immunoblotting of the cleavage / integrity status of pro-caspase 3, pro-caspase 8, pro-caspase 9 and the conformational change in BAX (after prior immunoprecipitation), 24h after drug exposure; *Right section*; cells were treated with PD98059 and UCN-01 as described for the left section, except that 24h after treatment, cells were isolated, fixed, digested with RNAase, stained with propidium iodide and flow cytometric analyses performed to assess cell cycle progression and the percentage sub-G1 DNA fragment content of MDA-MB-231 cells. The percentage of cells in each phase of the cell cycle was determined in duplicate from three separate experiments ± SEM. **Panel C.** Primary cell types were cultured as described in McKinstry *et al.* [22]. Cells were treated for 48h with Vehicle (DMSO), 10 μM PD184352, 150 nM UCN-01 or the drug combination. Cells were isolated by trypsinization 48h after treatment, fixed to glass slides, and stained for double stranded DNA breaks using a TUNEL assay kit. The percentage apoptosis was determined in triplicate from two separate experiments ± SEM.

Figure 2. PD184352 and UCN-01 combine to suppress the growth of established estrogen-dependent MCF7 mammary carcinoma tumors in a greater than additive fashion. Estrogen pellets were implanted in the subscapular midline of female athymic mice and one week after implantation, animals were injected with 5 million MCF7 cells in 100 μl reduced growth factor Matrigel subcutaneously into the rear flank; the tumor take rate was ~60%. Tumors were permitted to form over the following month. Animals with palpable tumors (~150 mm^3) were sorted with the intention of providing a normal distribution of tumor volume within each group, and so that the mean tumor volume of all animals within a group was within 10%. Animals were injected with Vehicle, PD184352, UCN-01 or the drug combination for 2 days as described in the Methods section. Tumors were callipered to determine their volume calculated as ($width^2$) x length / 2, where width is the smaller of the two measurements. The mean tumor volume for all animals in each treatment condition was plotted ± SEM (n = 8). Data are from a representative of three independent studies.

Figure 3. PD184352 and UCN-01 combine to suppress the growth of established estrogen-independent MDA-MB-231 mammary carcinoma tumors in a greater than additive fashion. Animals were injected 10 million cells in 50 μl PBS female athymic mice subcutaneously into the rear flank: tumor take rate was over 90%. Tumors were permitted to form over the following 20 days. Animals with palpable tumors (~100 mm^3) were sorted with the intention of providing a normal distribution of tumor volume within each group, and so that the mean tumor volume of all animals within a group was within 10%. Animals were injected with Vehicle, PD184352, UCN-01 or the drug combination for 2 days as described in the Methods section. Tumors were callipered to determine their volume calculated as ($width^2$) x length / 2, where width is the smaller of the two measurements. The mean tumor volume for all animals in each treatment condition was plotted ± SEM (n = 6 for PD184352, UCN-01 and PD184352+UCN-01 and n = 5 for vehicle control. One of the sorted vehicle control tumors did not proliferate over the time course of the study and was excluded from our data set and statistical calculations). Data are from a representative of four independent studies.

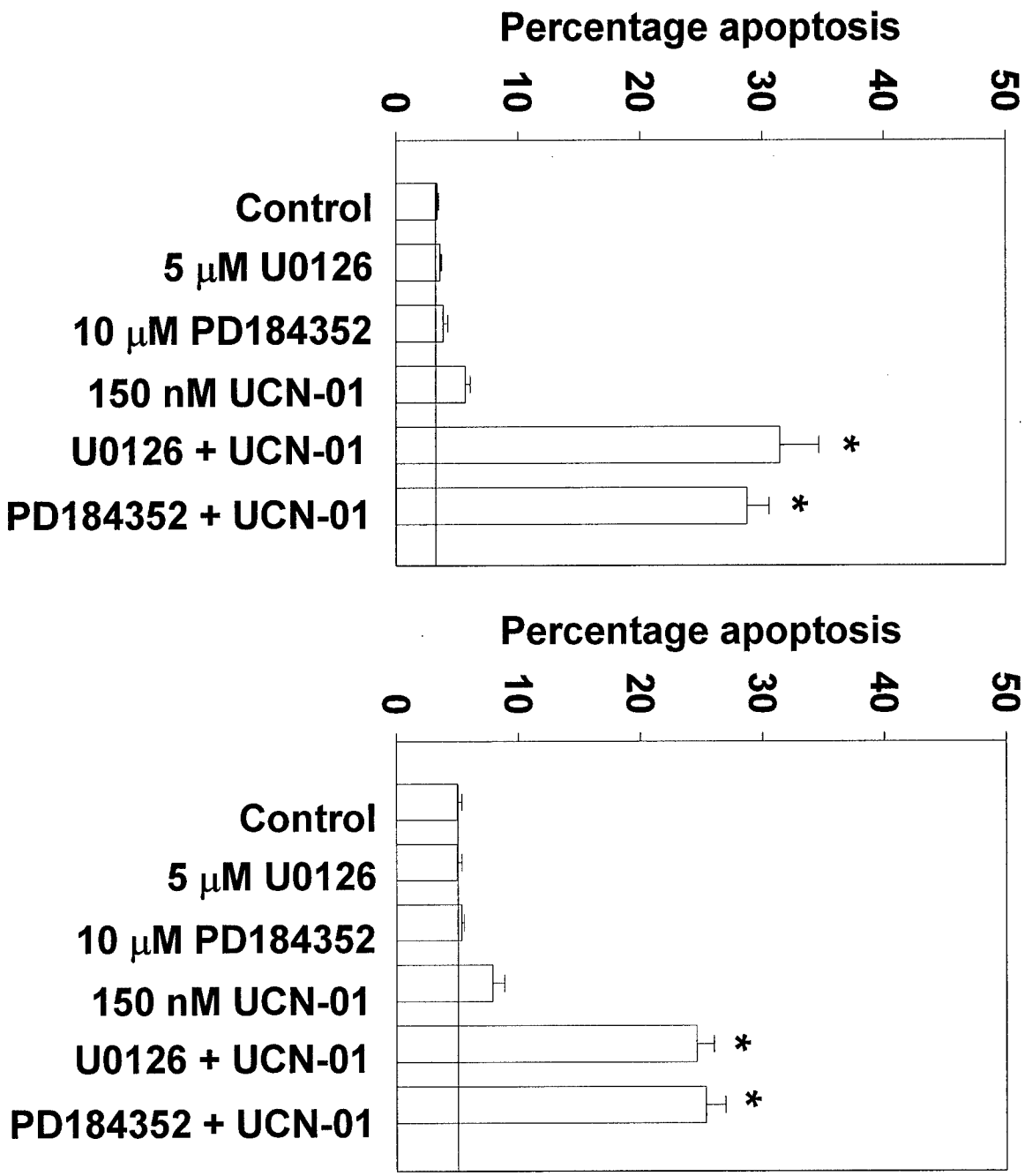
Figure 4. PD184352 and UCN-01 combine to suppress the cellularity of established estrogen-independent MCF7 mammary carcinoma tumors 30 days after drug exposure which correlates with suppression of ex vivo colony formation. Animals with MCF7 flank tumors were injected with Vehicle, PD184352, UCN-01 or the drug combination for 2 days as described in the Methods section. Tumors were recovered from animals 32 days after the start of drug exposure. Isolated tumors were divided into two equal parts; one portion of the tumor was macerated and digested to obtain single cells that were plated in vitro to determine the ex vivo colony forming ability of the treated tumor cells, as described in the Methods [18, 22]. Survival data are normalized and presented as the impact of PD184352 on the tumor cell colony formation of Vehicle or UCN-01 treated tumors, whose survival were each defined as 1.00; the non-normalized data value for treatment with UCN-01 was 0.68 ± 0.1. Data are from three independent tumors, each performed in triplicate ± SEM. The other portion of the tumor was fixed and 10 μm sections taken for H&E and immunohistochemical staining to determine tumor morphology and cellularity, and Ki67 and CD31 immunoreactivity. Images, except where indicated, were taken at 60x magnification using a Olympus microscope. Data are representative of images from three independent tumors.

Figure 5. PD184352 and UCN-01 combine to suppress the cellularity of established estrogen-independent MDA-MB-231 mammary carcinoma tumors 30 days after drug exposure which correlates with suppression of ex vivo colony formation. Animals with MDA-MB-231 flank tumors were injected with Vehicle, PD184352, UCN-01 or the drug combination for 2 days as described in the Methods section. Tumors were recovered from animals 32 days after the start of drug exposure. Isolated tumors were divided into two equal parts; one portion of the tumor was macerated and digested to obtain single cells that were plated in vitro to determine the ex vivo colony forming ability of the treated tumor cells, as described in the Methods [18, 22]. Survival data are normalized and presented as the impact of PD184352 on the tumor cell colony formation of Vehicle or UCN-01 treated tumors, whose survival were each defined as 1.00; the non-normalized data value for treatment with UCN-01 was 0.81 ± 0.06. Data are from three independent tumors, each performed in triplicate ± SEM. The other portion of the tumor was fixed and 10 μm sections taken for H&E and immunohistochemical staining to determine tumor morphology and cellularity, and Ki67 and CD31 immunoreactivity. Images, except where indicated, were taken at 60x magnification using a Olympus microscope. Data are representative of images from three independent tumors.

Figure 6. Combined PD184352 and UCN-01 exposure causes a rapid induction of MDA-MB-231 tumor cell death within 2 days of drug treatment. Animals with MDA-MB-231 flank tumors were injected with Vehicle, PD184352, UCN-01 or the drug combination for 2 days as described in the Methods section. Tumors were recovered daily from animals 1-5 days after the start of drug exposure. Isolated tumors were fixed and 10 μm sections taken to determine: **Panel A**, H&E staining for tumor morphology and cellularity for days 1-3; **Panel B**, H&E staining for tumor morphology and cellularity for days 4-5; **Panel C**, immunohistochemical staining for ERK1/2 phosphorylation; **Panel D**, immunohistochemical staining for ERK1/2 phosphorylation after exposure to UCN-01 from a control image, a day 1 image and a day 3 image. **Panel E**, immunochemical staining for CD31 reactivity to indicate endothelial cells. Images, except where indicated, were taken at 60x magnification using a Olympus microscope. Data are representative of data from three independent tumors.

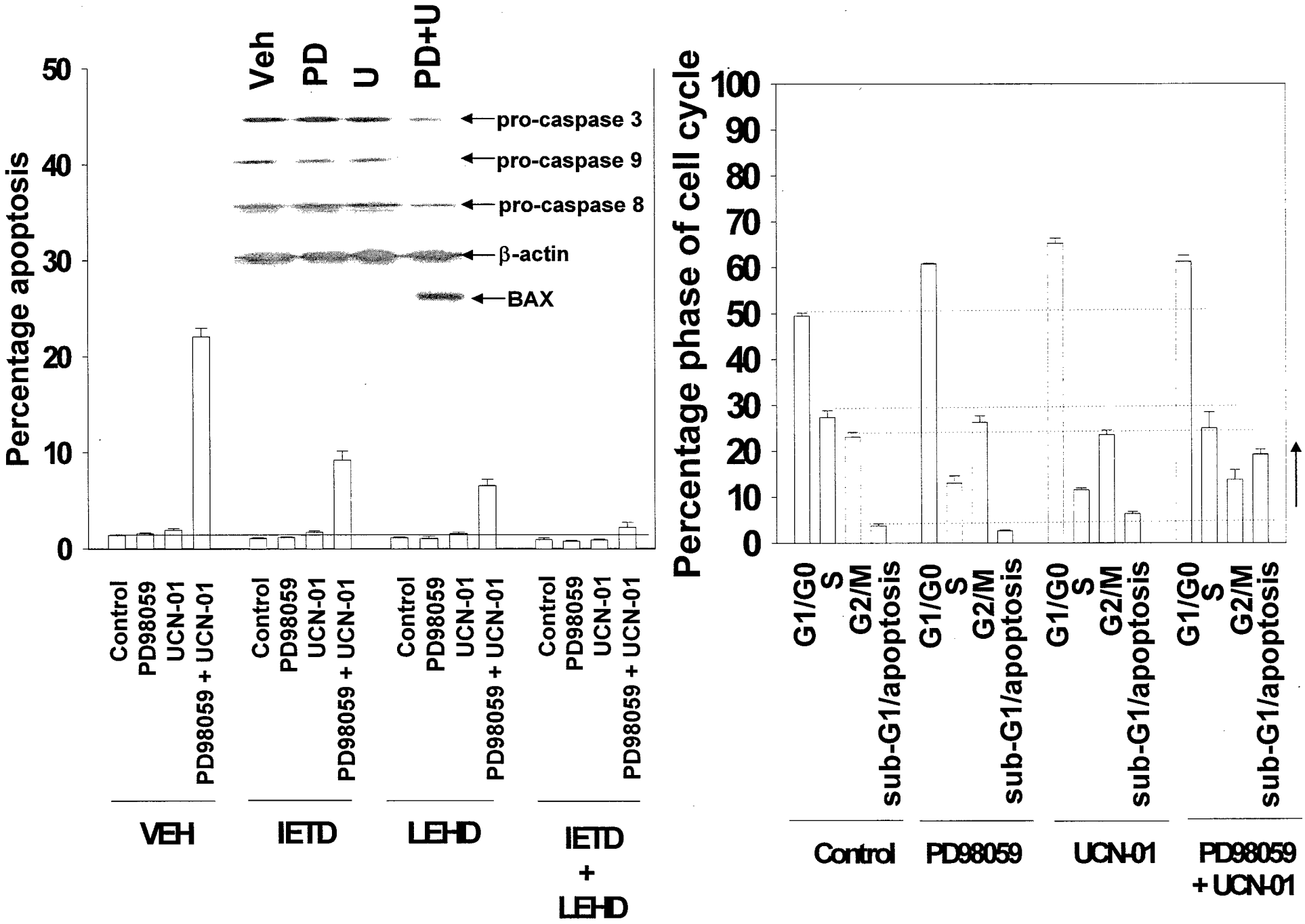
Figure 7. Combined PD184352 and UCN-01 exposure causes a prolonged reduction of MDA-MB-231 tumor cellularity, ERK1/2 phosphorylation, Ki67 and CD31 immunoreactivity that is maintained for over 20 days post-drug treatment. Animals with MDA-MB-231 flank tumors were injected with Vehicle, PD184352, UCN-01 or the drug combination for 2 days as described in the Methods section. Tumors were recovered from animals 5-20 days after the start of drug exposure. Isolated tumors were fixed and 10 μm sections taken to determine: **Panel A**, H&E staining for tumor morphology and cellularity; **Panel B**, immunohistochemical staining for Ki67 immunoreactivity; **Panel C**, immunohistochemical staining for ERK1/2 phosphorylation; **Panel D**, immunohistochemical staining for CD31 immunoreactivity at days 5 and 15. Images, except where indicated, were taken at 60x magnification using a Olympus microscope. Data are representative of data from three independent tumors.

Figure 8. UCN-01 exposure causes a prolonged activation of ERK1/2 in tumors containing MCF7 cells, but not in tumor derived from MDA-MB-231 cells. Animals with MDA-MB-231 flank tumors were injected with Vehicle, PD184352, UCN-01 or the drug combination for 2 days as described in the Methods section. Tumors were recovered from animals 32 days after the start of drug exposure. Isolated tumors were fixed and 10 μm sections taken for immunohistochemical staining to determine ERK1/2 phosphorylation. Images, except where indicated, were taken at 60x magnification using a Olympus microscope. Data are representative of images from three independent tumors.

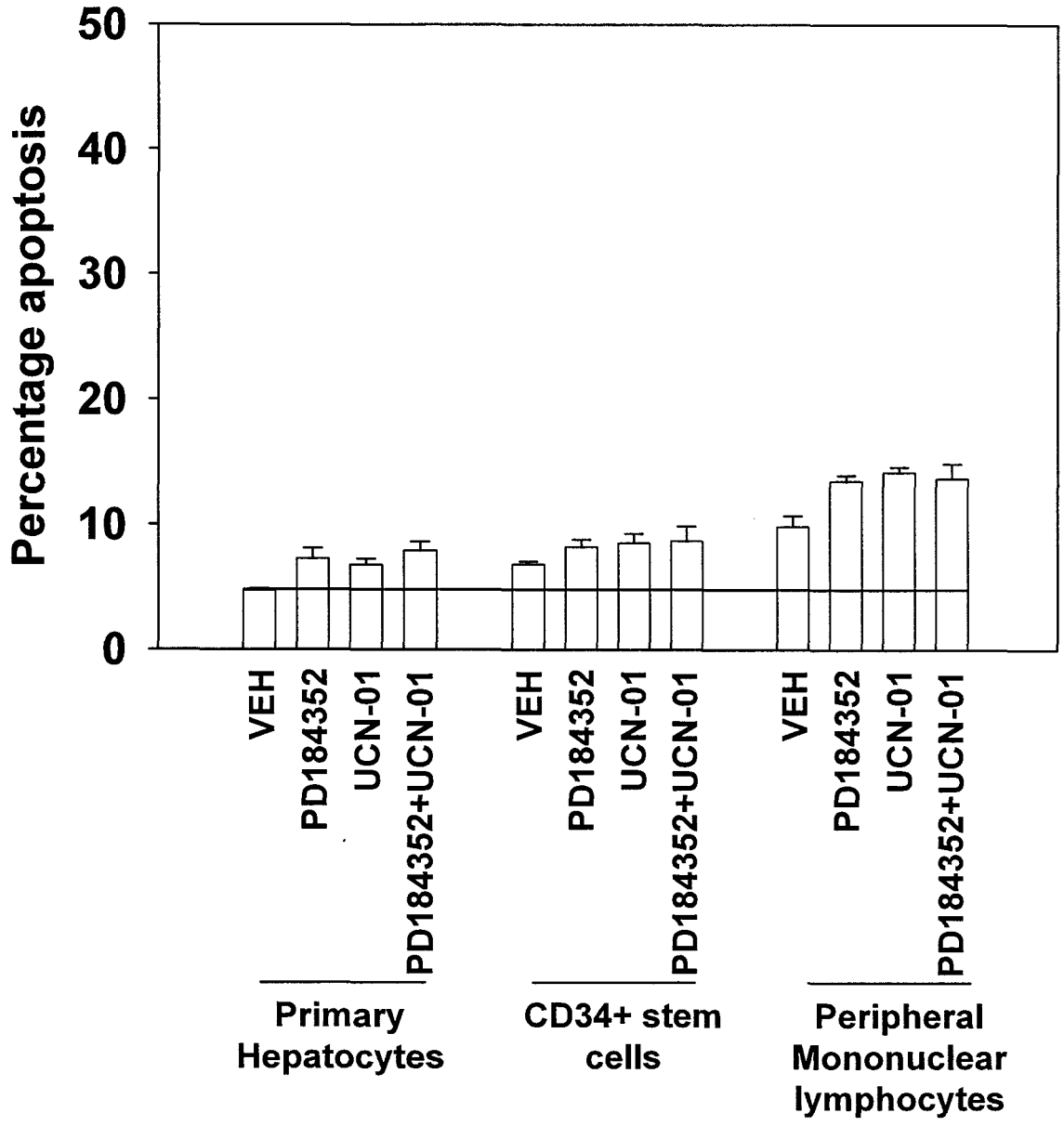


Hawkins et al. Figure 1A

Hawkins et al. Figure 1B



Hawkins et al. Figure 1C



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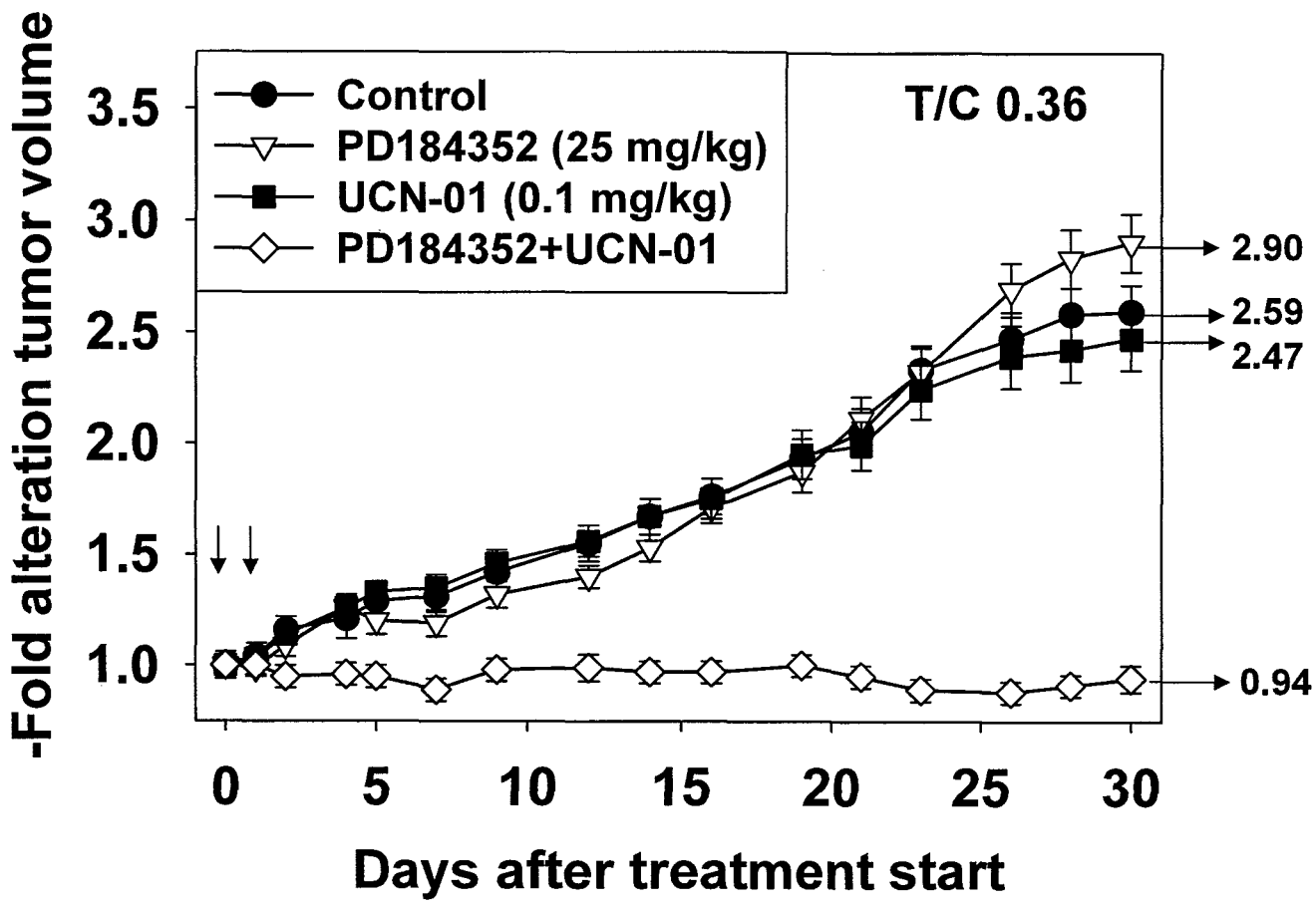

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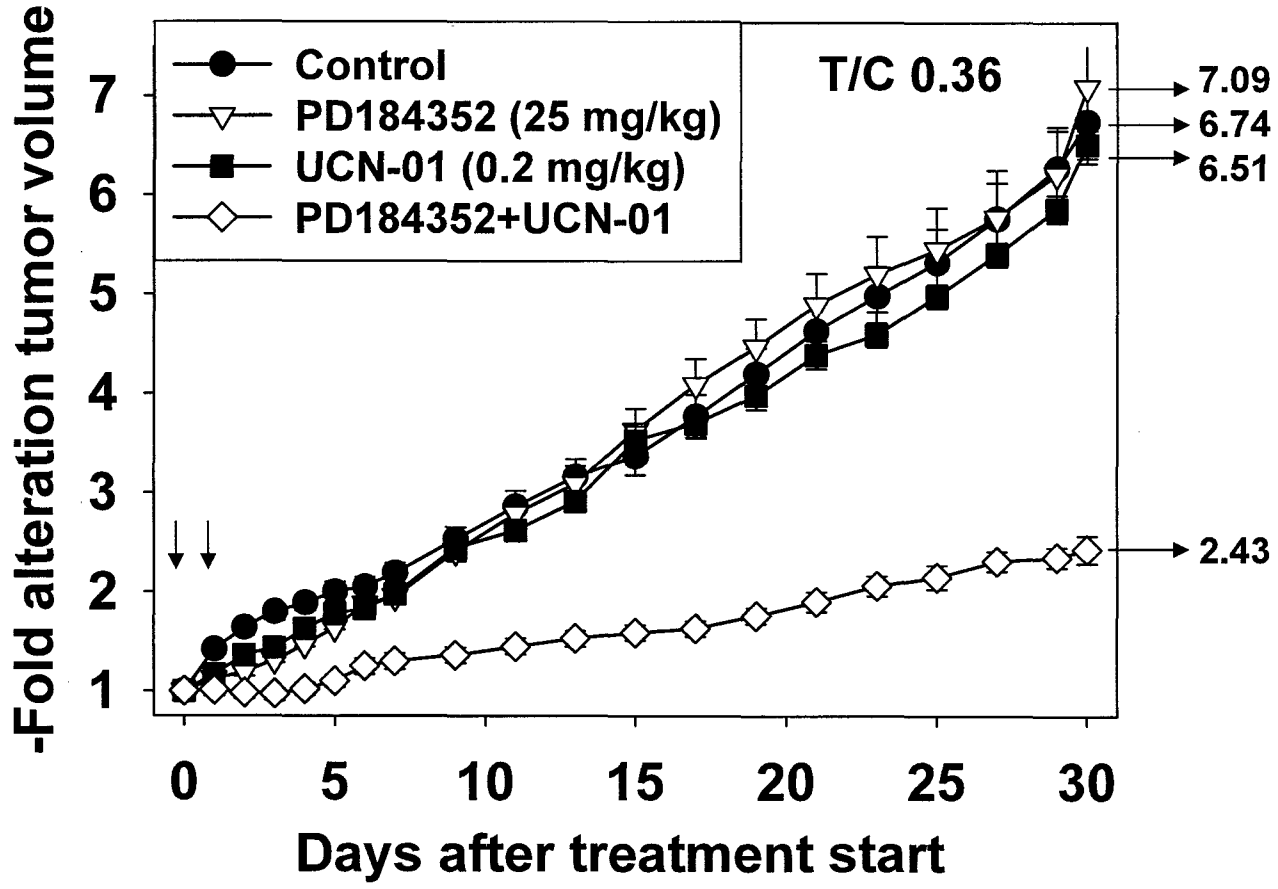

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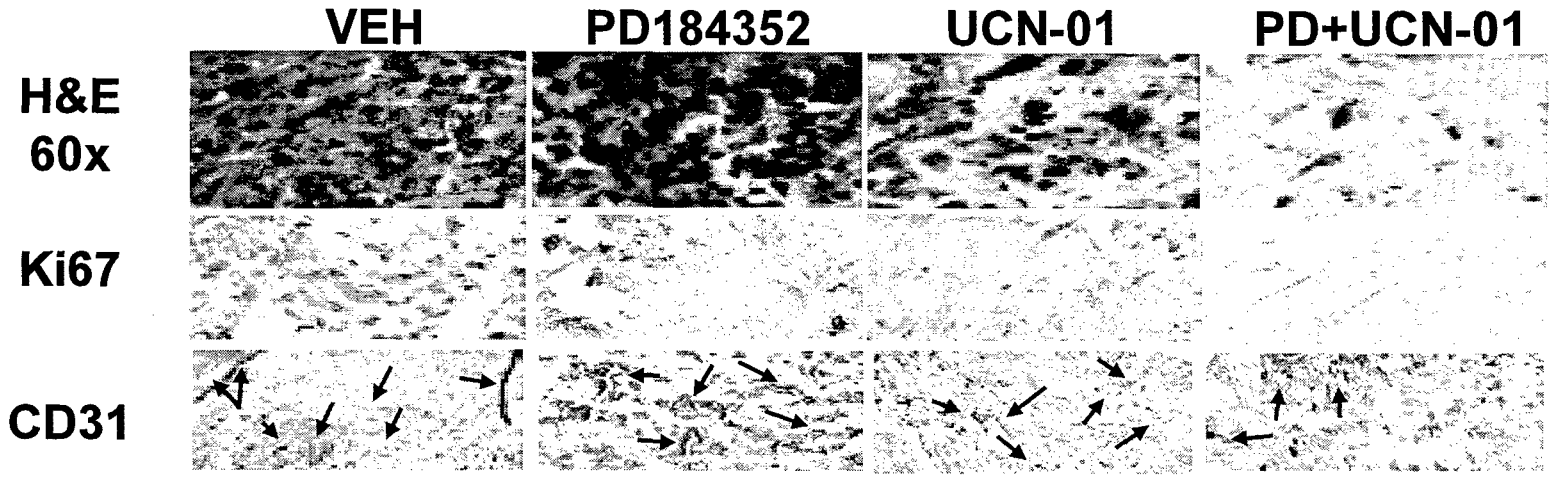

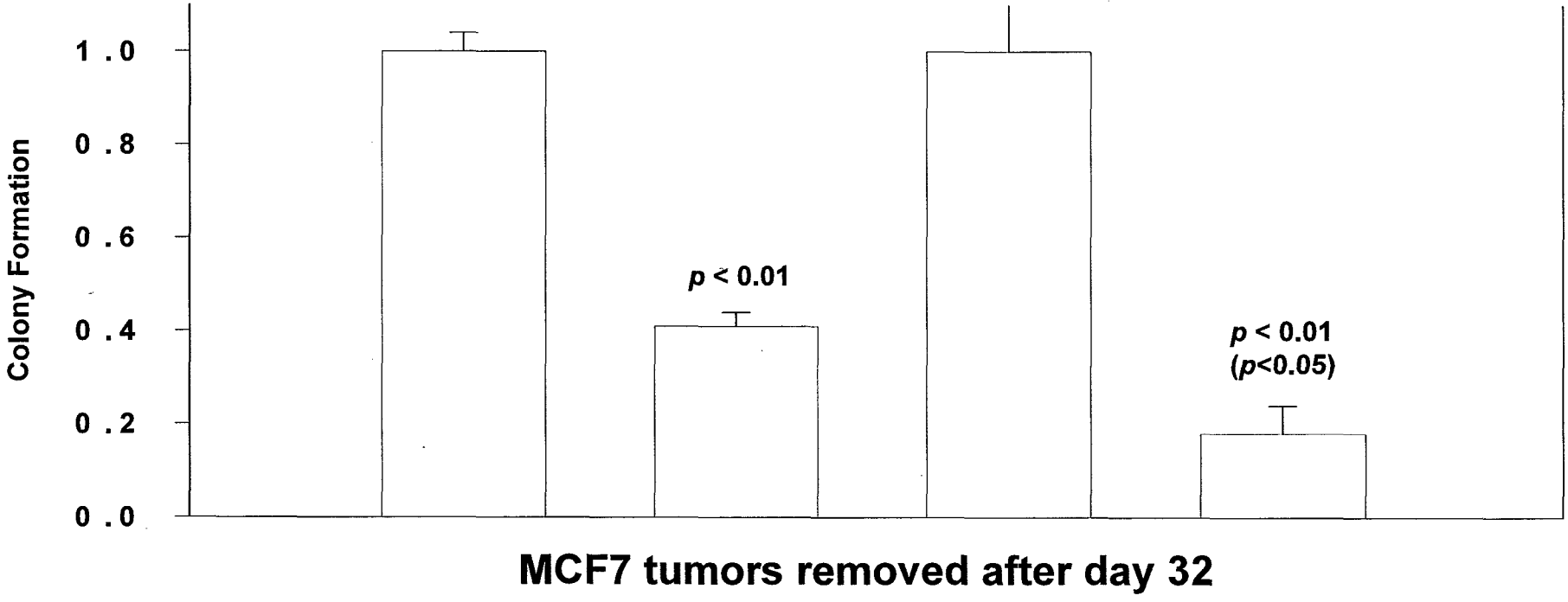

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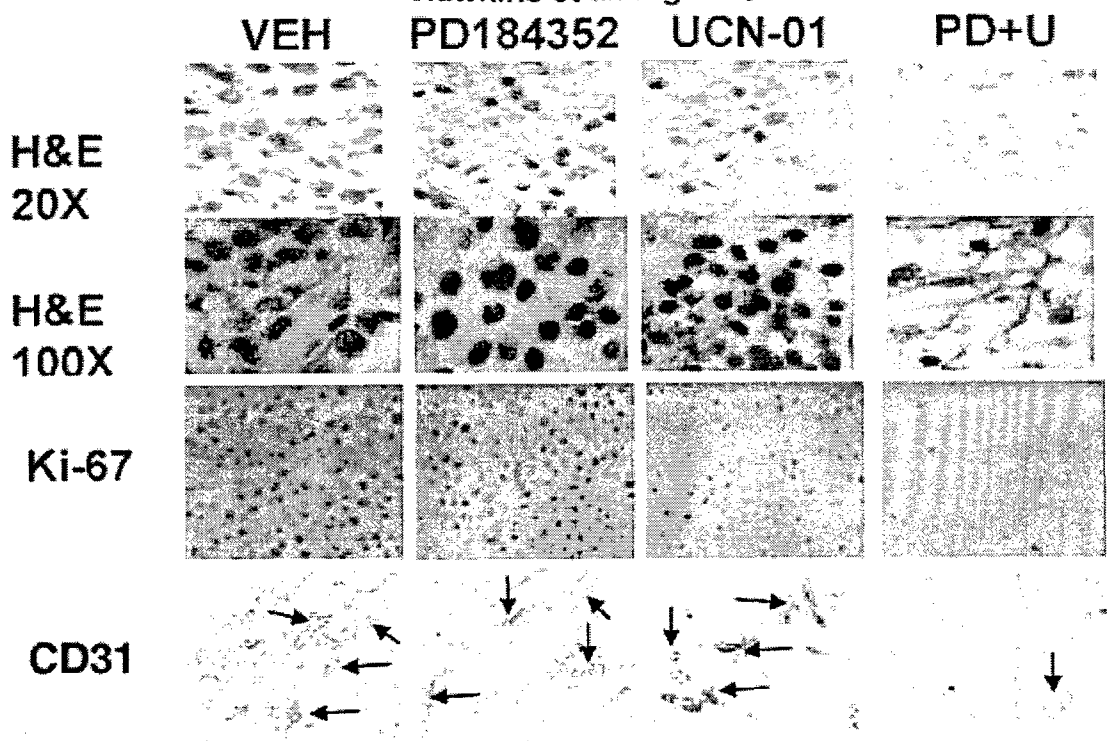

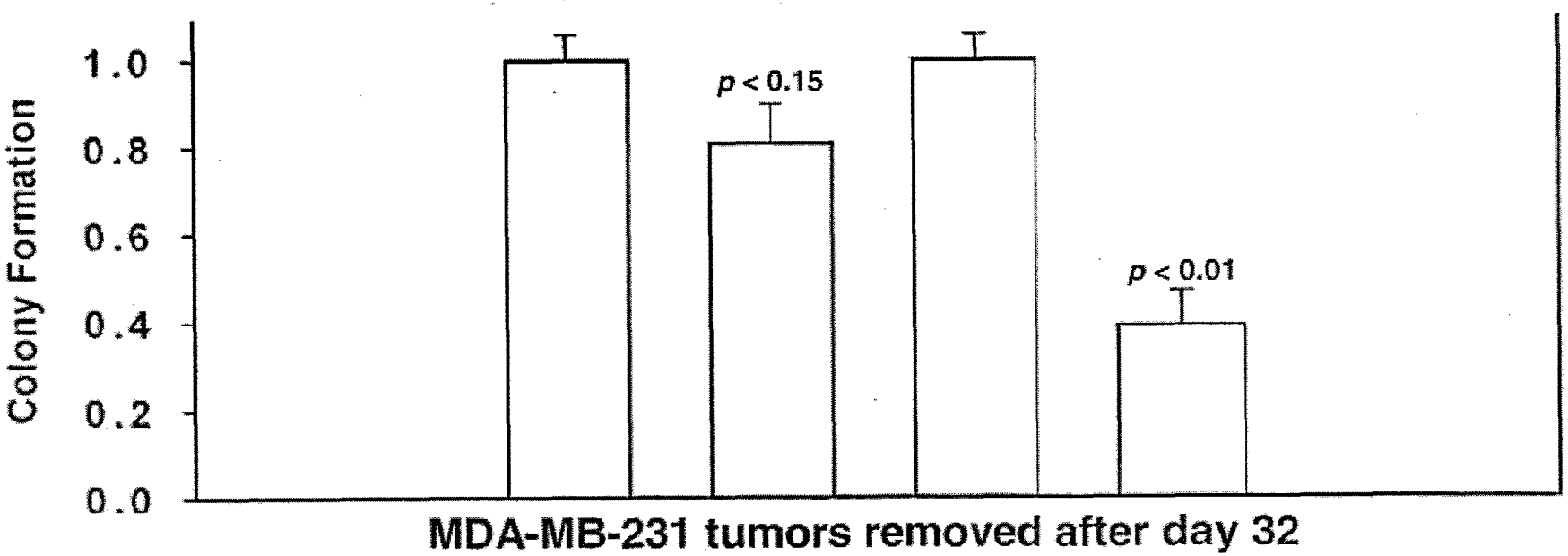

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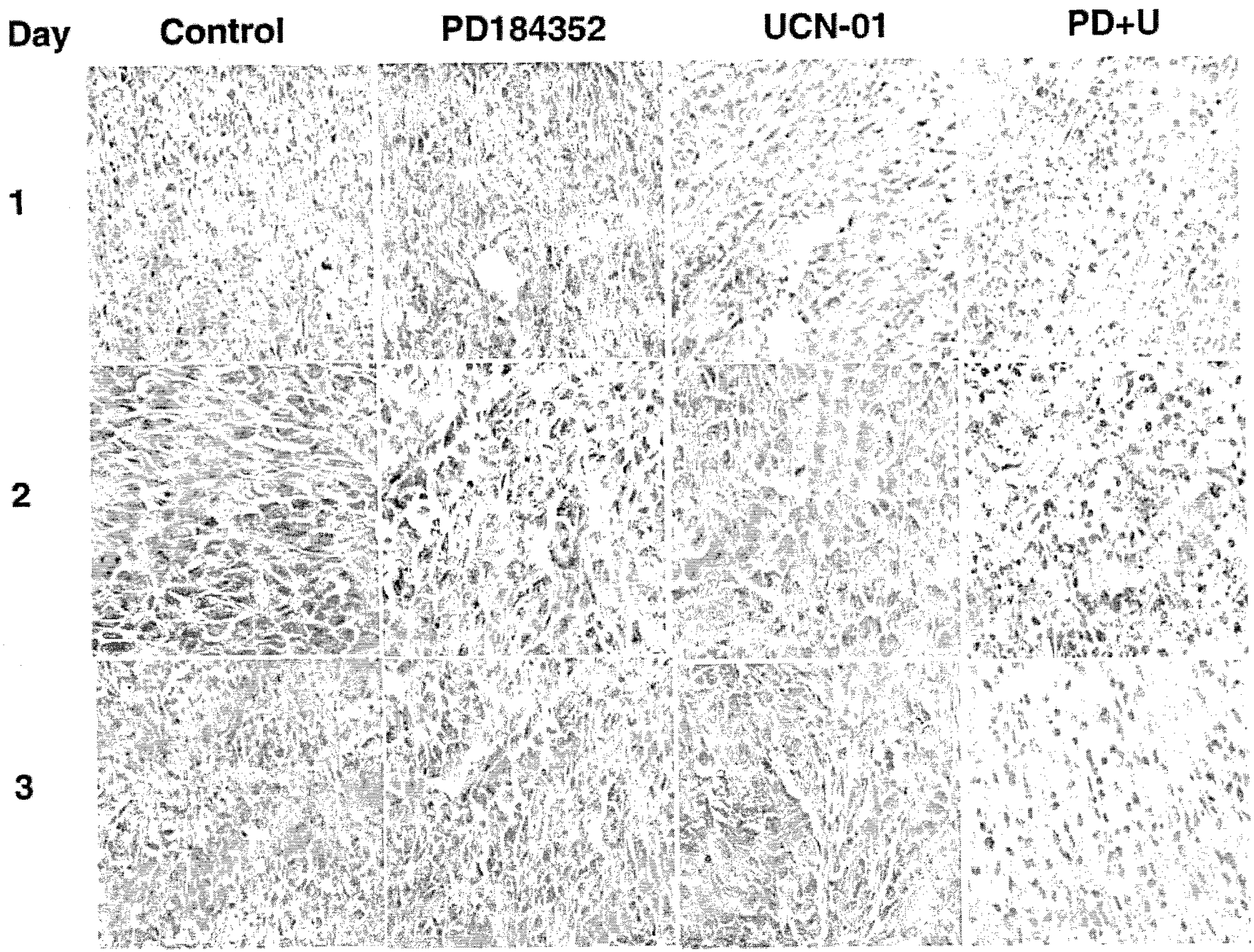

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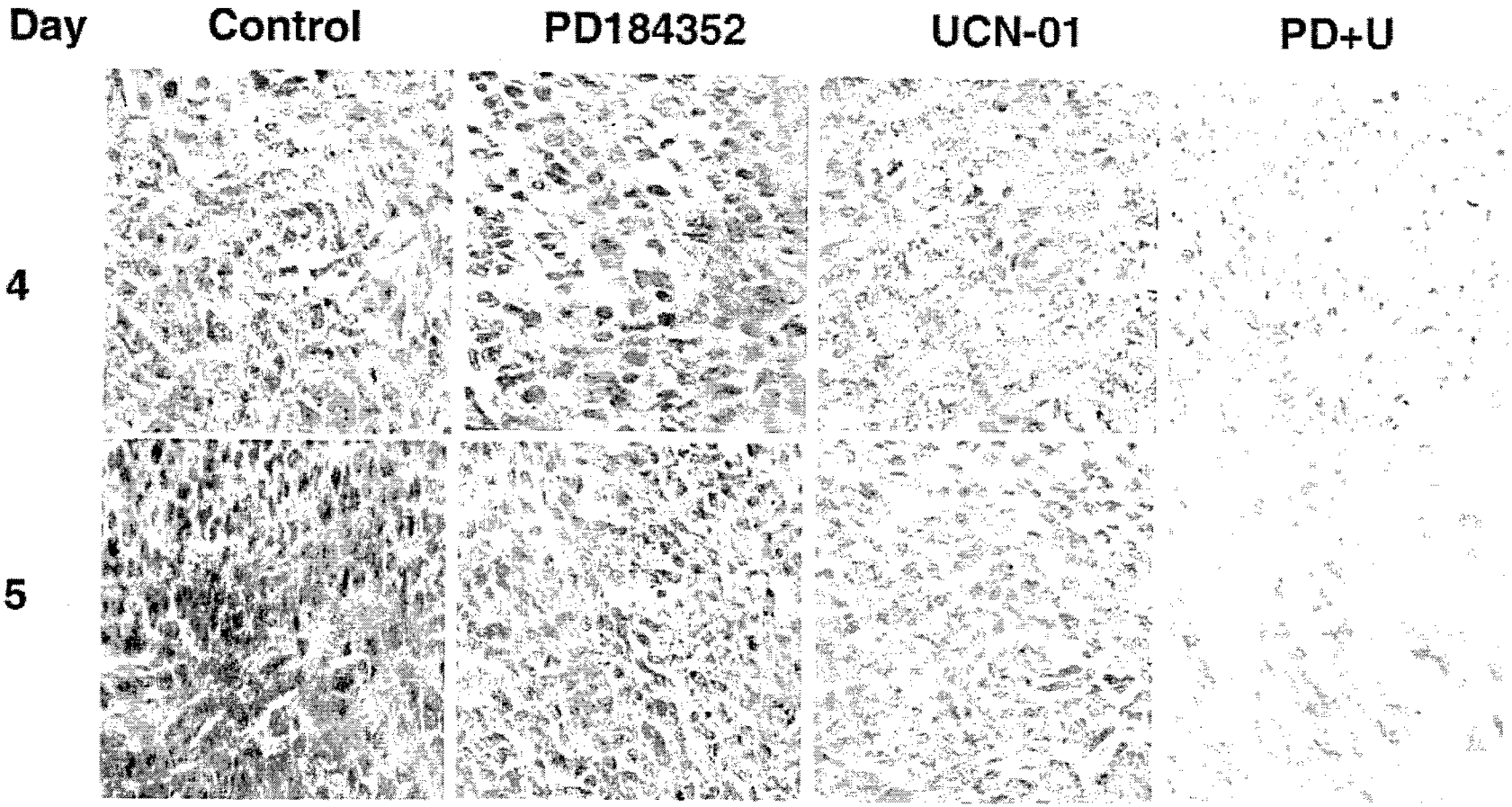

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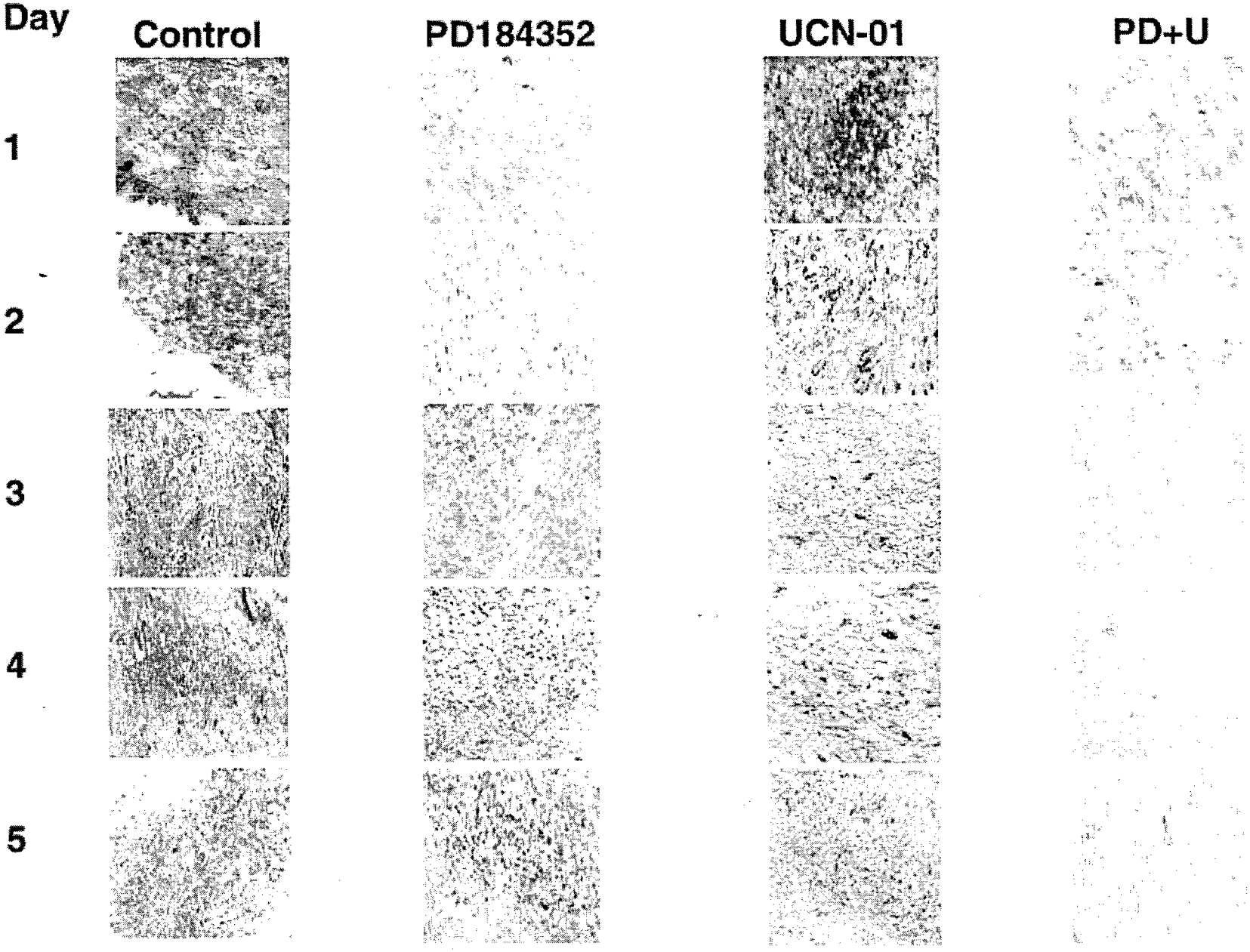

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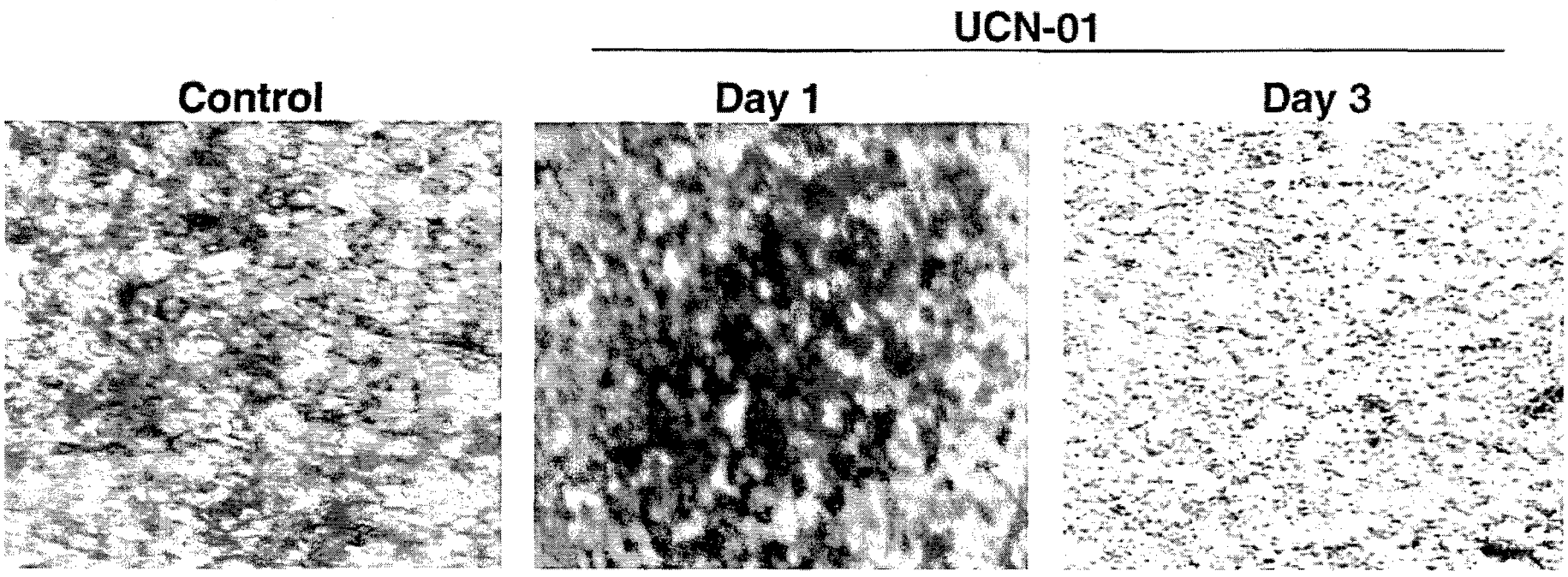

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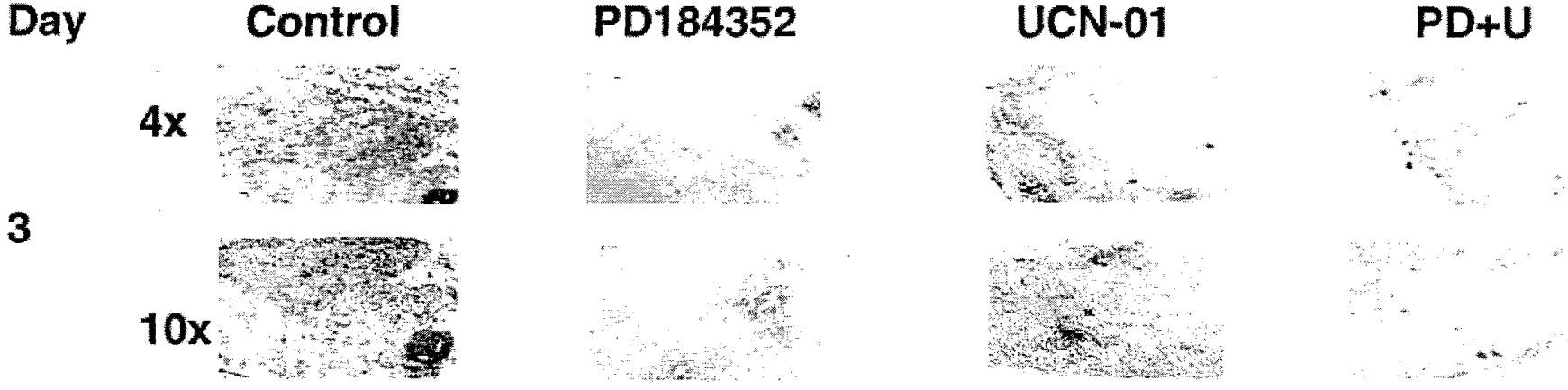
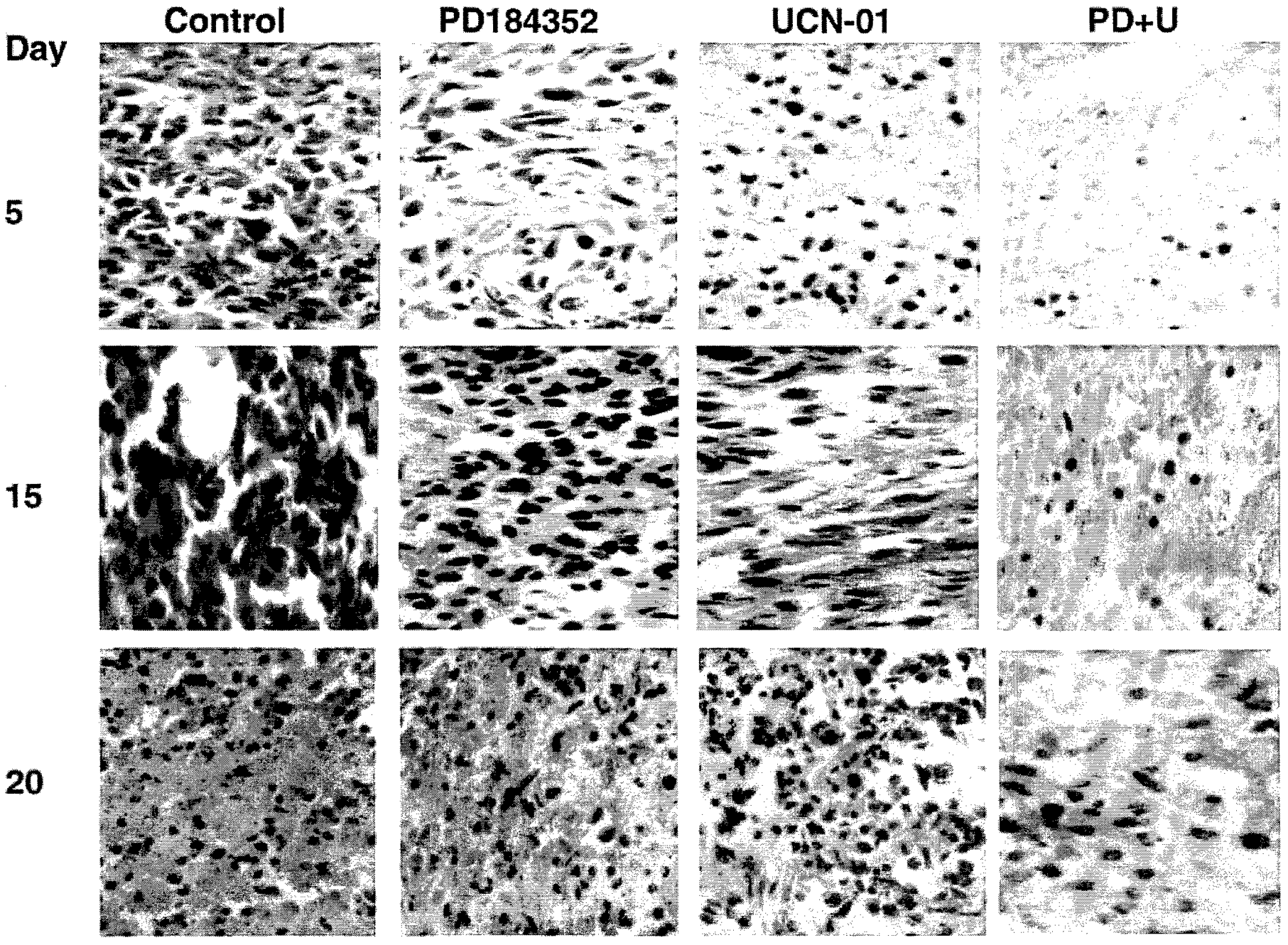


Hawkins et al. Figure 7A



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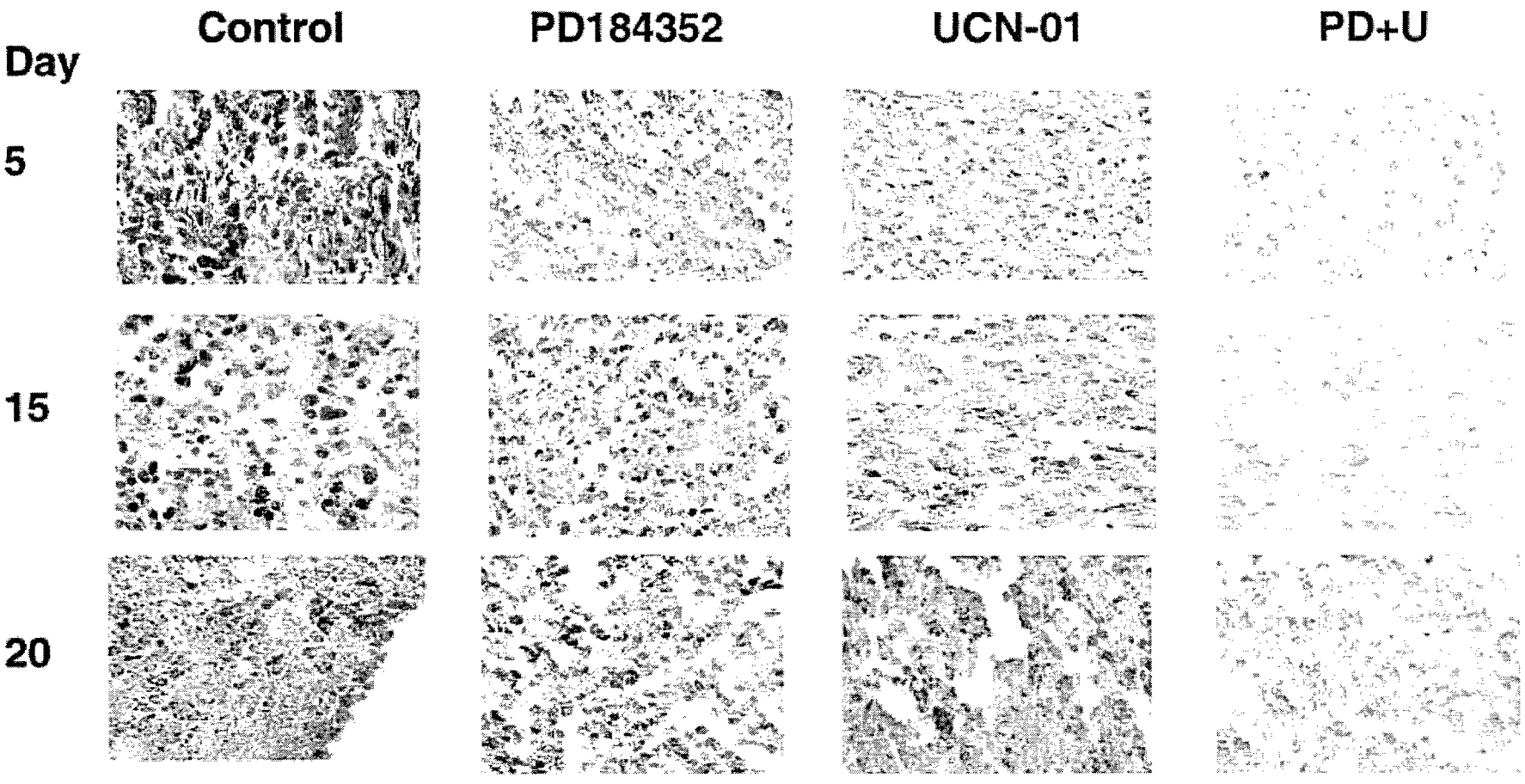

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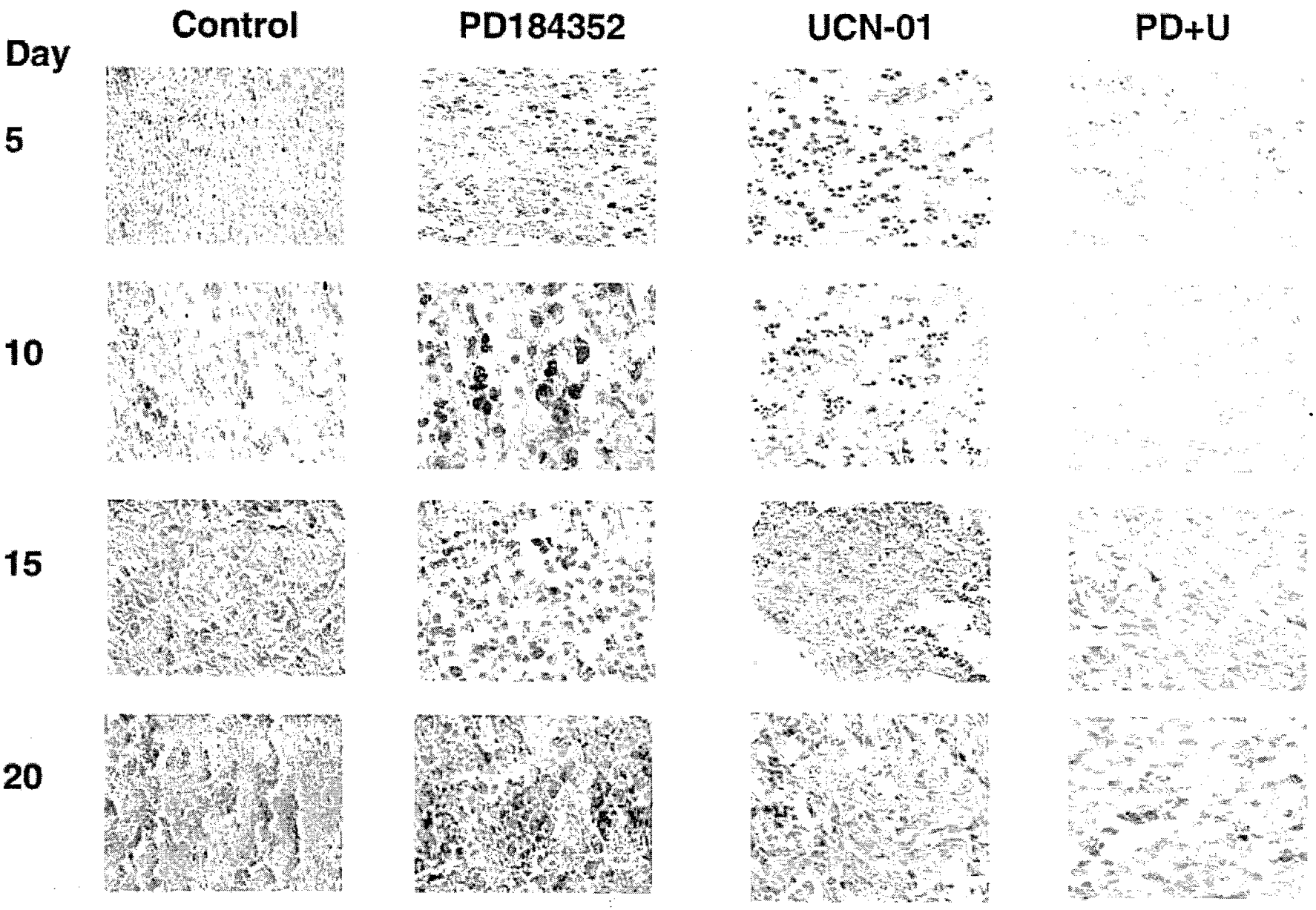

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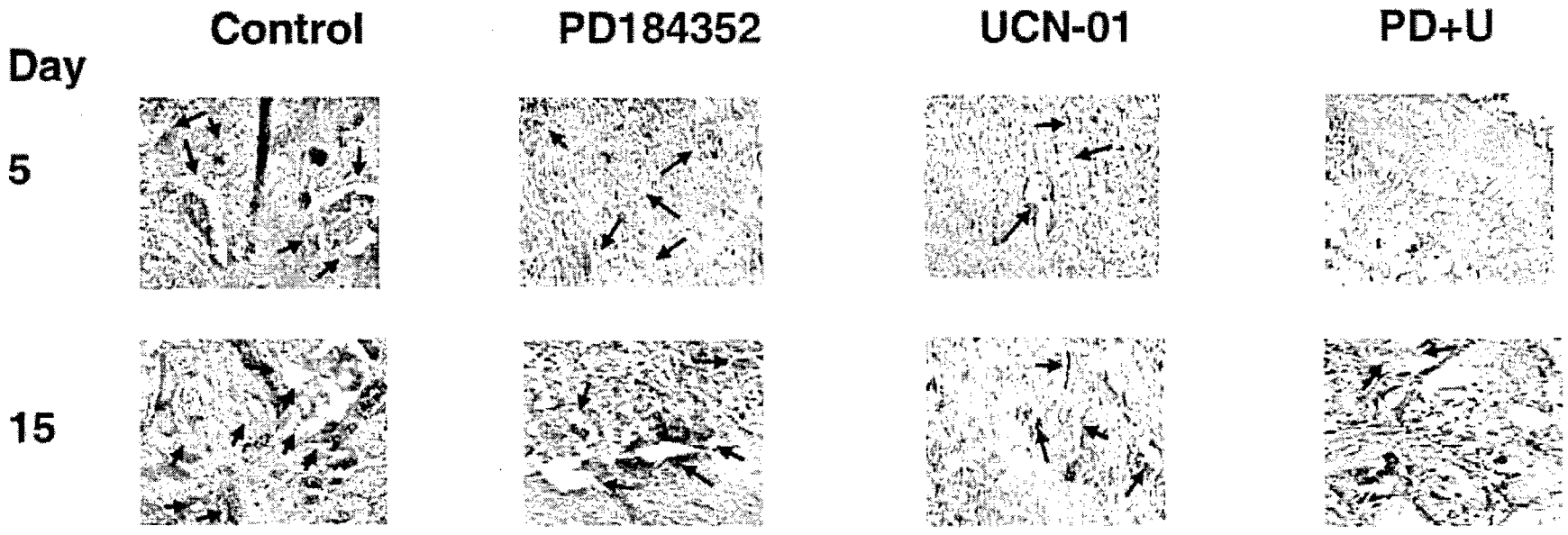

Hawkins et al. Figure 8